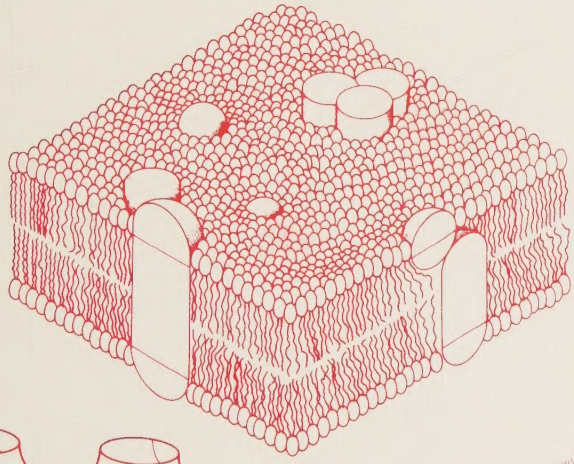
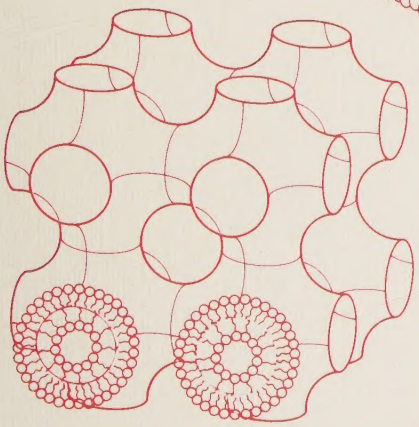
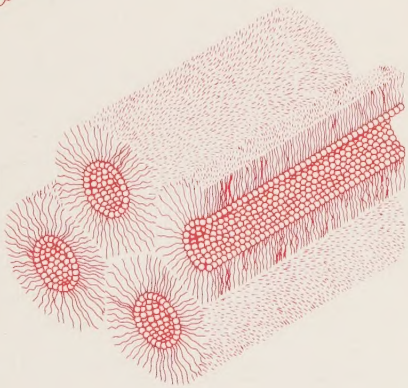
REGULATION AND PHYSICAL PROPERTIES OF BACTERIAL MEMBRANE LIPIDS

Leif Rilfors







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av

Leif Rilfors

Fil.kand., Mlm.

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Abstract

The regulation of acyl chain composition and polar head group composition in the membranes of *Acholeplasma laidlawii* and *Bacillus megaterium* was investigated. Membrane lipid composition is influenced by the growth temperature, by incorporation of cholesterol into the membranes, and by the fatty acid content of the growth medium. From physiological and physico-chemical investigations of membrane lipids with branched acyl chains, it is concluded that iso and anteiso acyl chains act like straight saturated and unsaturated acyl chains, respectively, in membranes containing large amounts of these components.

Investigations of the phase equilibria in lipid-water systems were carried out with the predominant lipids from the two bacterial membranes. Phase equilibria in these systems are affected by: (1) the structure of the polar head group; (2) the structure of the acyl chains; (3) temperature; and (4) the presence of cholesterol.

The lipid regulation mechnisms occurring in the two bacterial membranes are interpreted in terms of geometric and packing properties of the individual lipid species, and not in terms of membrane "fluidity". It is concluded that the effective shape of the lipid molecules is an important factor governing the regulation of membrane lipid composition in *A. laidlawii* and *B. megaterium*.

Key words *Acholeplasma laidlawii*, *Bacillus megaterium*, lipid composition, monoglucosyldiglyceride, diglucosyldiglyceride, phosphatidylethanolamine, cholesterol, branched acyl chains, cubic phase, reversed hexagonal phase, molecular shape

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PAPERS I-VI

This thesis is based on the following publications, referred to in the text by their Roman numerals, and on some unpublished results.

I. Lipid Bilayer Stability in Membranes. Regulation of Lipid Composition in *Acholeplasma laidlawii* As Governed by Molecular Shape. Å. WIESLANDER, A. CHRISTIANSSON, L. RILFORS & G. LINDBLOM (1980) Biochemistry 19:3650-3655.

II. The Effect of Cholesterol on the Phase Structure of Glucolipids from *Acholeplasma laidlawii* Membranes. A. KHAN, L. RILFORS, Å. WIESLANDER & G. LINDBLOM (1981) Eur. J. Biochem. 116:215-220.

III. Reversed Cubic Phase with Membrane Glucolipids from *Acholeplasma laidlawii*. ^{1}H, ^{2}H, and Diffusion Nuclear Magnetic Resonance Measurements. Å. WIESLANDER, L. RILFORS, L.B.-Å. JOHANSSON & G. LINDBLOM (1981) Biochemistry 20:730-735.

IV. Lipid and Protein Composition of Membranes of *Bacillus megaterium* Variants in the Temperature Range 5 to 70°C. L. RILFORS, Å. WIESLANDER & S. STÄHL (1978) J. Bacteriol. 135:1043-1052.

V. Cubic Liquid Crystalline Phase with Phosphatidylethanolamine from *Bacillus megaterium* Containing Branched Acyl Chains. L. RILFORS, A. KHAN, I. BRENTEL, Å. WIESLANDER & G. LINDBLOM (1982) FEBS Lett., in press.

VI. Membrane Lipid Composition in *Acholeplasma laidlawii* A Grown on Iso and Anteiso Branched-Chain Saturated Fatty Acids. L. RILFORS, submitted to Eur. J. Biochem..

INTRODUCTION

The elucidation of structure and function of biological membranes has been the subject of a huge number of investigations during this century. In 1925, Gorter and Grendel proposed that a lipid bilayer constitutes the basic structure of erythrocyte membranes (1). About ten years later Danielli and Davson interpreted surface tension measurements and lipid-protein interaction studies to indicate that the lipid polar head groups of cell membranes are covered with two layers of protein: one flat extended layer intimately bound to a lipid bilayer, and one outer layer of loosely attached globular proteins (2,3). As early as 1941, the existence of a bilayer structure was convincingly demonstrated by X-ray diffraction studies of myelin (4) and a variety of hydrated lipids from natural sources (5). Electron microscopy investigations of several biological membranes and pure lipid vesicles revealed a triple-layered structure about 75 Å thick (6). These results, together with the X-ray diffraction data, supported the concept that a lipid bilayer is the core of biological membranes. However, the bilayer structure remained controversial until around 1970 (7), when high resolution X-ray diffraction measurements unambiguously demonstrated the presence of this structure in various membranes (8-10). The commonly accepted membrane model today is the so called Singer & Nicolson fluid mosaic model (11): the proteins are wholly or partly embedded in a fluid-like bilayer matrix, and are able to diffuse more or less freely in two dimensions. Cell membranes are thus regarded as flexible and dynamic structures. However, the interaction between membrane components and the thermodynamics of lipid and protein self-assembly are not treated in this model (12). The different lipid species present in a biological membrane are not assigned individual properties, and

no attempt is made to explain why some lipids form bilayers in isolation while others do not. Most biological membranes contain at least one major lipid species that in isolation forms a non-bilayer structure under physiological conditions (13). It can thus be expected that the membrane lipids are acting not only as a fluid matrix. They probably have other structural and functional tasks as well.

Among physical chemists working with amphiphiles such as detergents and soaps, it has been known since long that these substances, when mixed with water, self-assemble to aggregates with different shapes (14). It was suggested almost thirty years ago that the geometry of these amphiphiles is an important factor determining the aggregate shape (15). Lipids isolated from biological membranes were also shown to form different aggregate types (16,17), and lipid molecular geometry was proposed by Luzzati and coworkers to be the reason for variations of the aggregate shape in these systems as well (18,19). Attempts to *quantify* the aggregation process and to explain the formation of different aggregate types have been performed by Tanford (20) and Israelachvili and coworkers (12,21). In these calculations intermolecular interactions and the thermodynamics of self-assembly are taken into account. Different lipid species aquire distinct packing properties, depending on the chemical structure of the polar head group and the hydrocarbon chains (see section "Self-assembly of amphiphiles" for details). The packing of proteins in a lipid bilayer is also considered, indicating that the packing of lipids and proteins is higly coupled (22).

About sixty years ago it was suggested that the melting point of the cellular fat determines the temperature at which a cell dies (23).

The idea that the temperature at which the lipids solidify is the minimum temperature for growth was also presented (24). It was early noticed that the melting point of cellular lipids is directly proportional to the growth temperature of the organism, and that the degree of unsaturation of the lipids is decreased at higher temperatures (25). Over the past years, changes in the degree of acyl chain unsaturation have appeared to be a commonly used mechanism for membrane lipid regulation in organisms subjected to variations in the ambient temperature, procaryotic as well as eucaryotic (26-29). Differential scanning calorimetry and X-ray diffraction studies revealed that the "melting" of membrane lipids in a bilayer structure actually is an endothermic transition from a gel state with closely packed and fully extended hydrocarbon chains, to a liquid crystalline state with a disordered and liquid-like hydrocarbon region (30,31). Since the transition temperature increases with a decreasing degree of hydrocarbon chain unsaturation (32), it has been proposed that organisms regulate the ratio saturated/unsaturated chains, in response to the growth temperature, in order to maintain the membrane lipid "fluidity" (or "microviscosity") at a certain level (33) or within a certain range (34). However, a general control of the position of the phase transition temperature interval in relation to the growth temperature cannot be required, since the bacterium *Acholeplasma laidlawii* can grow isothermally having an acyl chain composition ranging from totally polyunsaturated (VI) to almost fully saturated (35). In some microorganisms the end point of the gel to liquid crystalline transition interval is 30-40°C below the growth tempenature; on the other hand, good growth is obtained even when about 50% of the membrane lipids are in the gel state (36). The interpretation of the concept fluidity is also doubtful.

It is a *macroscopic* quantity defined for a *three-dimensional* system, with no obvious extension to a two-dimensional microscopic system (37). The fluidity is directly proportional to the diffusion coefficient for a three-dimensional system, but for an anisotropic fluid such as a lipid bilayer, no simple relation between these quantities exists (37). When the fluidity of membranes is experimentally determined, the measured values often contain both dynamic and static information (38-40), and erroneous conclusions concerning the connection between fluidity and various membrane functions may be drawn. Instead of fluidity more well-defined physicochemical quantities should be used, such as correlation times and translational or rotational diffusion coefficients to describe molecular motions, and order parameters to describe the molecular structure (37). An example of the confusion caused by the concept fluidity is the effect of cholesterol incorporation into a lipid bilayer in the liquid crystalline state. Cholesterol is claimed to decrease the bilayer "fluidity" in this case (36). However, the order parameter of the acyl chains is markedly increased, but practically no change is observed in the lateral and rotational diffusion coefficients of the lipids (37). There is consequently no connection between static and dynamic quantities in a lipid bilayer.

In this thesis the lipid regulation mechanisms occurring in two bacterial membranes are interpreted in terms of geometric and packing properties of individual lipid species. Two model organisms, *Acholeplasma laidlawii* and *Bacillus megaterium*, have been used. These organisms have qualitative differences in polar head group composition as well as in acyl chain composition. The major membrane lipids have been isolated from the organisms grown under different conditions, and

studies of the phase equilibria in lipid-water systems support the presented hypothesis.

SELF-ASSEMBLY OF AMPHIPHILES

The thermodynamics of self-assembly of amphiphiles into micelles, and the role of molecular geometry for this process, has been treated by several authors (15,20,21,41). The self-assembly theory was recently further developed to explain the formation of vesicles, extended bilayers, and lipid aggregates of the reversed type ("water-in-oil" aggregates) (12,42). This theory involves: (1) the interaction free energies of the amphiphiles; (2) the geometry of the molecules; and (3) a thermodynamic treatment of the whole system. The self-association of amphiphiles in aqueous solution is accounted for by the "hydrophobic effect": the contact between the water molecules and the hydrophobic part of the amphiphiles tends to be eliminated, and the interfacial area between the water phase and the hydrocarbon phase is decreased (20). The hydrophobic effect dictates a lower limit to the aggregate size, because a minimum number of amphiphiles must associate with each other in order to effectively eliminate the hydrocarbon-water contact. Repulsive forces are also involved in the formation of amphiphilic aggregates. They tend to increase the interfacial area between the water phase and the hydrocarbon phase, and they set an upper limit to the aggregate size. Repulsive interactions emerge from electrostatic repulsion between head groups, hydration forces, and steric head group and hydrocarbon chain interactions (12,20,43). In the absence of the repulsive forces the amphiphiles would form an entirely separate phase instead of dispersed aggregates. The opposing

forces result in an optimal surface area per head group at the hydrocarbon-water interface (a), at which the total interaction free energy per lipid molecule is at a minimum. Geometric considerations must now be applied to determine the most favoured aggregate structure in which the interaction free energy is minimized and the entropy is optimized (12). Besides the hydrocarbon-water interfacial area, two quantities are used to describe the geometric or packing properties of amphiphiles: the hydrocarbon chain volume (v) and the hydrocarbon chain length (l). When these quantities are specified for a given lipid, it is possible to determine the size and shape of the aggregate that the lipids can pack into while the values of the quantities are kept constant. If these values can be satisfied by several different aggregate structures, entropy will favour the structure with the smallest aggregation number (12).

A packing parameter for each amphiphile is defined by the three geometric quantities: $v(a \cdot l)^{-1}$. The value of the packing parameter determines the structure of the amphiphilic aggregate (12). When $v(a \cdot l)^{-1} < 1/3$ spherical aggregates are formed; $v(a \cdot l)^{-1} = 1/3 - 1/2$ results in rod-like ("oil-in-water") aggregates; lamellae are formed when $v(a \cdot l)^{-1} = 1/2 - 1$; and aggregates of the reversed type ("water-in-oil") form if the packing parameter exceeds 1. These aggregates can build up different liquid crystalline phase structures. Liquid crystalline lipid phases are characterized by a "liquid" state of the hydrocarbon part of the molecules, while there simultaneously exists a superior long-range order in one, two or three dimensions, as in crystals. The phases with a one-dimensional lattice are formed by lamellar aggregates (44). Lamellae and infinitely long rods can be packed in two-dimensional lattices. Some biological membrane lipids

are able to form such phases, viz. the normal ("oil-in-water", H_{I}) and reversed ("water-in-oil", H_{II}) hexagonal phases, which are composed of rods (II,45-48). The cubic phases, with three-dimensional lattices, can be built up by all the three kinds of aggregates: spheres, rods and lamellae (49,50). In resemblance to the hexagonal phases, the cubic phases can be of the normal (Q_I) or the reversed (Q_{II}) type. Cubic phases can also be formed by biological membrane lipids (II,III,V,17,51-54).

The packing properties of lipids are influenced by the chemical structure of the molecules, as well as by different extrinsic factors. Some of the factors affecting the value of the packing parameter are: the structure of the polar head group and the acyl chains, temperature, and the presence of cholesterol.

(1) With a given acyl chain composition, i.e., constant v and l, any change in the size (bulkiness, charge, hydration) of the *polar head group* will affect the optimal surface area (a) of the lipid molecule. Modifications of polar head structure can thus alter the value of $v(a \cdot l)^{-1}$.

(2) For lipids with a given polar head group (constant a), the molecular shape depends on several factors. A decreased ratio of saturated/cis-unsaturated *acyl chains* reduces the length of the hydrocarbon chains (l) and increases the width of the hydrocarbon space almost without any change in the hydrophobic volume (v) (I). By increasing the *temperature*, trans-gauche isomerizations and 2g1 kinks are introduced into the hydrocarbon chains, yielding the same effects as mentioned above. When the ratio iso/anteiso acyl chains is reduced, l is probably not altered. Instead v increases since anteiso chains cannot be as closely packed as iso chains (V,55). All these changes thus

increase the value of the packing parameter.

(3) *Cholesterol* is a flat, rigid lipid molecule, with a small surface area and a large hydrophobic volume (56), and it cannot form bilayers at physiological temperatures (57). It can be predicted that this sterol, due to its effective wedge-shape (56), promotes the formation of non-lamellar lipid phases. This effect is actually observed when cholesterol is mixed with monounsaturated glycerolipids (II,54,58). Cholesterol thus *increases* the value of $v(a \cdot l)^{-1}$. The sterol molecule also has another effect on the glycerolipids: the order parameter of the acyl chains is increased (37), and the number of trans-gauche isomers is reduced in these chains (59). This effect of cholesterol *decreases* the value of the packing parameter. The latter effect seems to dominate when cholesterol is mixed with glycerolipids containing large amounts of polyunsaturated acyl chains: the temperature for the transition from a lamellar (L) to an H_{II} phase for human erythrocyte phosphatidylethanolamine (PE) and egg yolk PE is increased by cholesterol (58).

In a living cell, the cytoplasmic membrane must be able to separate the interior of the cell from its surroundings. Thus, the bilayer structure, i.e., the L phase, is the only lipid structure compatible with a functioning biological membrane. If lipids forming both lamellar and non-lamellar phases are present in a membrane, a balance between these lipids must be maintained, and cells must be able to actively regulate the balance if it is disturbed by different factors.

MODEL ORGANISMS

The suitability of *Acholeplasma laidlawii* as model organism for membrane studies depends above all on two factors: (1) the possibility of introducing controlled alterations in the acyl chain composition of the membrane lipids; and (2) the ease with which the cell membrane can be isolated and purified, due to the lack of cell wall and intracellular membranes. Several fundamental aspects of membrane structure and function have been clarified by using *A. laidlawii*. A gel to liquid crystalline phase transition in a biological membrane was first demonstrated with this organism (30,31). It was also used to prove that a lipid bilayer is the predominant structural component of cell membranes (9,31). Investigations of the connection between the physical state of the membrane lipids and various membrane functions such as permeability, and transport and enzymatic activities, have also been made with *A. laidlawii* (60,61).

A. laidlawii strain A (EF22) (35) has been used in the present studies. This organism is capable of synthesizing straight-chain and branched-chain saturated fatty acids from short-chain precursors such as acetate (62), 2-methylbutanoic acid, and 3-methylbutanoic acid (VI). Strain A has an absolute growth requirement for straight-chain unsaturated, cyclopropane, or branched-chain fatty acids (35,63,VI). Five polar lipids always occur in the membranes of *A. laidlawii* A (35): monoglucosyldiglyceride (MGDG), diglucosyldiglyceride (DGDG), phosphatidylglycerol (PG), and glycerophosphoryl derivatives of MGDG and DGDG. Two other polar lipids occur under certain growth conditions. The first, glucolipid X (GLX), is synthesized in increasing amounts by the organism when the fraction of straight saturated acyl chains in the membrane lipids is increased (35,62,64,65). GLX has been

suggested to be composed of two diglyceride molecules linked to one glucose molecule (35,66). The other, diphosphatidylglycerol (DPG), is synthesized constantly when the organism is grown on linoleic acid (VI), and is synthesized temporarily after a temperature shift from 37°C to 17°C, when the organism is grown on oleic acid (64). Some of the lipid biosynthetic pathways in *A. laidlawii* are known (67), and a tentative scheme of these pathways is presented in paper I.

Bacillus species are gram-positive microorganisms, and the cell membrane is surrounded by a thick peptidoglycan cell wall. In particular, the species *B. megaterium* and *B. subtilis* have been utilized in a large number of cell membrane and cell wall studies. The structure, synthesis and turnover of the cell wall have been investigated (68-70), and the activities of several membrane-bound enzymes have been determined (71-73). Many studies have dealt with the interaction between the chromosome and the cell membrane (74,75). Since both mesophilic and thermophilic species occur within the genus *Bacillus*, investigations of the phenomenon of thermophily have been performed with these species (76-78).

The membrane lipids of *Bacillus* species contain mainly iso and anteiso methyl-branched saturated acyl chains (79,IV). Iso acids have their branching point at the penultimate carbon atom while anteiso acids have this point displaced one carbon atom towards the carboxyl group. These fatty acids were first isolated from wool fat (80) and from different kinds of animal fats (81). In 1960, it was reported that these branched acyl chains constitute the major acyl chain fraction in membrane lipids from *B. subtilis* and a *Sarcina* species (82,83). Iso and anteiso fatty acids have now been found to occur in large amounts in nine genera of gram-positive bacteria and four

genera of gram-negative bacteria (79). The chain initiators in the fatty acid synthesis are branched short-chain acids: isobutyrate, 3-methylbutyrate, and 2-methylbutyrate. These acids are in turn derived from valine, leucine, and isoleucine, respectively. As with most organisms, malonyl-CoA serves as chain extender and NADPH as hydrogen donor (79). The most commonly occuring polar membrane lipids in *Bacillus* are PE, PG, and DPG (79,IV). The lipid biosynthetic pathways in *B. megaterium* are a matter of controversy. On one hand it is claimed that the pathways in this organism are the same as those in *Escherichia coli*, i.e., PG and PE are synthesized via two different branches from a common intermediate (84). On the other hand, pulse-chase experiments suggest that PE is synthesized from PG (85).

Due to the greater fatty acid synthesizing capacity of *B. megaterium*, its membrane lipid composition is not as readily controllable as that of *A. laidlawii*. Moreover, the cell wall of *B. megaterium* protects and stabilizes the cell membrane, and this organism seems to be less sensitive to changes in the environment than *A. laidlawii*. For example, higher concentrations of both general and local anesthetics are needed to inhibit growth of *B. megaterium* as compared to *A. laidlawii* (86).

PHASE EQUILIBRIA OF MEMBRANE LIPID-WATER SYSTEMS

Investigations of the phase equilibria were carried out with the predominant lipids from the two bacterial membranes. The glucolipids MGDG and DGDG constitute 55-80% (mol/mol) of the polar membrane lipids in *A. laidlawii* A (I,VI,35,64,65), and PE makes up 55-70% (mol/mol) of the polar lipids in the membranes of the thermophilic *B. megaterium* strains (IV). The phase equilibria in the lipid-water systems are

affected by the factors altering the value of the packing parameter (see page 11): (1) the structure of the polar head group; (2) the structure of the acyl chains; (3) temperature; and (4) the presence of cholesterol (II,III,V). These factors are discussed below.

(1) *Polar head group*. DGDG with different acyl chain contents forms an L phase in excess water, while MGDG forms an H_{II} phase under the same conditions (II,III,47,54). MGDG has a considerably smaller surface area (a) than DGDG (cf. 46). When these lipids contain $\geqslant$ 95% (mol/mol) oleoyl chains, a lipid sample with an MGDG/DGDG ratio of 1.2 forms a mixture of two phases at 30°C, viz. an L phase and a reversed cubic (Q_{II}) phase (III). If the glucolipid ratio is increased to 2.5, a one-phase system is obtained consisting of a Q_{II} phase.

(2) *Acyl chains*. The impact of the acyl chain structure on the phase equilibria is evident with the glucolipid mixtures. When the lipids contain about equal amounts of palmitoyl and oleoyl chains an L phase is formed at 50°C by a mixture with an MGDG/DGDG ratio of 1.2 (III). If these lipids contain $\geqslant$ 95% (mol/mol) oleoyl chains a Q_{II} phase is obtained under the same conditions. The position of the methyl branching point in the acyl chains also influences the lipid phases that are formed. At low water contents and at 60°C PE containing a high ratio iso/anteiso acyl chains forms an L phase, while the lipid forms a Q_{II} phase with a tenfold lower ratio iso/anteiso acyl chains (V). Investigations of the translational diffusion coefficient of the lipids in the Q_{II} phases show that these coefficients are either of the same order of magnitude or about one order of magnitude lower than the lateral diffusion coefficient observed for lipids in an L phase (III,V). These results strongly indicate that the cubic phases are bicontinuous, i.e., they are built up of continuous hydrocarbon regions as well as of continuous water regions.

(3) *Temperature*. When the temperature is increased the tendency of lipid-water mixtures to form Q_{II} and H_{II} phases also increases (II,V). This holds for single lipids and lipid mixtures containing acyl chains of different structures. The transition is observed in the presence of cholesterol as well.

(4) *Cholesterol*. The addition of cholesterol to MGDG/DGDG mixtures containing $\geqslant$ 95% (mol/mol) oleoyl chains promotes the formation of non-lamellar phases (II). The Q_{II} phase is not formed with the cholesterol concentration used in this investigation, but the L phase is directly transformed into the H_{II} phase when the temperature is increased (see Figure 2 in paper II).

It is concluded from these results that the factors predicted to increase the value of the packing parameter actually increase the tendencies of lipid-water mixtures to form non-lamellar phases. The phase equilibria in the membrane lipid-water mixtures are satisfactorily described by a qualitative application of the self-assembly theory. An interpretation of the membrane lipid regulation mechanisms in *A. laidlawii* and *B. megaterium* in terms of packing properties of the individual lipid species thus seems to be well motivated.

REGULATION OF ACYL CHAIN COMPOSITION

The acyl chain composition of the *A. laidlawii* A membrane lipids is altered by the growth *temperature* and by the incorporation of *cholesterol* into the membrane (I,VI,64). This regulation mechanism is operating when the organism is cultured on straight-chain saturated and cis-unsaturated fatty acids, as well as on saturated methyl-branched iso and anteiso fatty acids.

Cells grown on an equimolar mixture of palmitic and oleic acid decrease the ratio palmitoyl/oleoyl chains in the lipids when shifted from 37°C to 17°C (I,64). The average acyl chain length and the ratio iso/anteiso acyl chains are reduced at lower growth temperatures when *A. laidlawii* A is fed with the branched-chain fatty acids (VI).

Addition of cholesterol to an *A. laidlawii* A culture grown on an equimolar mixture of palmitic and oleic acid yields a higher incorporation of oleic acid, both at 37°C and after a shift to 17°C (I,64). Incorporation of cholesterol also lowers the ratio iso-C_{17}/anteiso-C_{17} in the membrane lipids (VI).

Another acyl chain regulation mechanism working isothermally has been observed in *A. laidlawii* A. Shorter acyl chains are synthesized when the organism is grown on the iso fatty acid precursor as compared to the anteiso fatty acid precursor, both at 27°C and 37°C (VI). The change in packing properties brought about by moving the methyl branching point one carbon atom downwards, is thus counteracted by the reduction of the acyl chain length.

When the regulation of acyl chain composition in the *B. megaterium* membrane was studied, four temperature range variants of this species were used (IV). Together these variants cover the growth temperature interval between 5°C and 70°C. The features observed in the *A. laidlawii* membrane are found in the *B. megaterium* membrane as well, although the *A. laidlawii* membrane is more homogeneous with respect to acyl chain composition. The ratio iso/anteiso acyl chains is about 12 times higher at 70°C than at 5°C, and the chain length parameter $(C_{16}+C_{17}+C_{18})/(C_{14}+C_{15})$ increases about 5 times when the growth temperature is raised within this interval (IV). The same regulation mechanisms have also been noticed in two temperature range variants of *B. megaterium* others

than those investigated in paper IV, and a shift in growth temperature from 22°C to 50°C when the facultatively thermophilic strain (Ft R32) is in the early logarithmic phase of growth, brings about the same changes in acyl chain composition.

The possibility to increase the degree of unsaturation at lower growth temperatures is also exploited. The obligately thermophilic strain (Ot 32) synthesizes a hexadecenoic acid when grown close to its minimum temperature (IV). Mass spectrometric and gas-liquid chromatographic investigations indicate that the facultatively psychrophilic (Fp 1), mesophilic (M 1), and facultatively thermophilic (Ft R32) strains synthesize unsaturated branched-chain fatty acids of chain length C_{16} and C_{17} at growth temperatures below 35°C. The amount of these acyl chains increases by lowering the growth temperature, and they make up about 10% (mol/mol) at 20°C (V), and about 20% (mol/mol) at 5°C. When these components are taken into account, the total amount of branched acyl chains in the *B. megaterium* membrane constitutes 75-95% (mol/mol).

A decrease in the growth temperature or incorporation of cholesterol into a membrane leads to a reduced value of the packing parameter for the glycerolipids (see pages 11 and 12). This value is increased by a reduction of the ratios saturated/unsaturated acyl chains and iso/anteiso acyl chains in the membrane lipids.

REGULATION OF POLAR HEAD GROUP COMPOSITION

A. laidlawii A regulates the polar head group composition upon (1) incorporation of different fatty acids; (2) changes in growth temperature; and (3) incorporation of cholesterol (I,VI,35). The two

most important effects on the polar head group composition caused by these factors are: a change in the molar ratio between the dominating lipids MGDG and DGDG, and variations in the balance between ionic and non-ionic lipids. The ionic lipid fraction consists of PG and glycerophosphoryl derivatives of MGDG and DGDG (I). In addition, the amounts of GLX are of interest. This lipid can make up 15% (mol/mol) under certain growth conditions (65).

(1) *Fatty acid incorporation.* Alterations in the structure of the incorporated fatty acids yield correlated responses in polar head group composition. The molar ratio MGDG/DGDG decreases when the ratios palmitoyl/oleoyl chains (I) and iso/anteiso acyl chains (VI) in the membrane lipids are reduced, and when shorter fatty acids are incorporated (VI). Concomitantly, the amount of ionic lipids is increased in the cases with the straight-chain fatty acids and the branched-chain fatty acid precursors, and the GLX synthesis is diminished (VI,35). The synthesis of this lipid is preferentially stimulated by incorporation of fatty acids that, when supplied *alone* to the medium, does not support growth of *A. laidlawii* A, e.g. palmitic acid and iso-C_{17} (VI).

The described changes in fatty acid incorporation results in an increased value of $v(a \cdot l)^{-1}$. The value is however reduced by a decrease in the MGDG/DGDG ratio since MGDG has a smaller surface area (a) than DGDG (cf. 46). The increase in the ionic lipid fraction may have two consequences: a reduction of the packing parameter since ionic lipids have large surface areas due to electrostatic repulsion between the head groups (87), and a conservation of the surface charge density and surface potential (I).

(2) *Growth temperature.* When the growth temperature is altered, the

value of the packing parameter can be regulated by a change in incorporation of fatty acids from the growth medium (see the preceding section). However, the parameter is regulated by a change in the polar head group composition as well. The decreased value of $v(a \cdot l)^{-1}$, due to a lower growth temperature, is counteracted by an increased ratio of MGDG/DGDG, both when the organism is grown on the straight-chain fatty acids (I) and on the branched-chain fatty acids (VI). The ionic lipid fraction is decreased when the incorporated fatty acids yield membrane lipids with a gel to liquid crystalline transition temperature interval close to the growth temperatures (47,64,88), i.e., with the supplements palmitic/oleic acid (I) and iso-C_{15}/iso-C_{17} (VI). A decrease in temperature through the phase transition temperature interval will decrease the lateral packing area of the lipids, and will consequently increase the surface potential (I). The cells respond by reducing the ionic lipid fraction, and the surface potential is lowered. When the growth temperature is well above the end point of the transition interval, practically no change occurs in the amount of ionic lipids. GLX synthesis is not affected by the growth temperature (VI).

(3) *Incorporation of cholesterol.* Cholesterol incorporation into membranes containing dioleoyl glycerolipids increases the value of $v(a \cdot l)^{-1}$ (see page 12). The cells respond by reducing the ratio MGDG/DGDG and by increasing the synthesis of ionic lipids (I). These two responses are faintly expressed when large amounts of palmitoyl chains are present in the lipids (Å. Wieslander, unpublished results). However, the bilayer destabilizing effect of cholesterol is very small in these lipid systems (II), and the responses are probably not demanded. If the lipids contain branched acyl chains the MGDG/DGDG ratio is either increased or unaltered (VI). It can be anticipated that cholesterol exerts

a small stabilizing effect on a lamellar phase composed of these lipids. This result may be explained by the different steric configurations of oleic acid and branched-chain fatty acids (see page 19 in paper VI). Thus, cholesterol has not a general effect on the packing properties of glycerolipids. This is supported by the finding that cholesterol lowers the temperature for the transition from an L to an H_{II} phase in mixtures with dioleoyl PE, while this transition temperature is increased when cholesterol is mixed with PE containing large amounts of polyunsaturated acyl chains (58).

In *B. megaterium* the greatest difference in polar head group composition is found between the mesophilic strain and the thermophilic strains (IV). The difference seems not to be induced by the growth temperature since this factor has a rather small effect on the membrane phospholipid composition in each strain.

DISCUSSION

The straight-chain saturated and unsaturated fatty acids have no doubt been payed the largest attention in physiological as well as physico-chemical studies of biological membranes. However, sufficient data concerning the properties of iso and anteiso fatty acids are now available to allow a comparison to be made between these fatty acids and the straight-chain fatty acids. Such a comparison is of biological relevance since straight saturated and unsaturated acyl chains on one hand, and iso and anteiso acyl chains on the other hand, often occur together in large amounts in biomembranes. Several experimental observations support the conclusion that iso and anteiso acyl chains act like straight-chain saturated and unsaturated acyl chains, respectively (IV).

Regulation of the ratio iso/anteiso acyl chains is consequently an alternative mechanism operating in cell membranes of organisms synthesizing very small amounts of straight-chain fatty acids. In the summary presented below a straight-chain saturated fatty acid (SFA) is compared to a straight-chain unsaturated fatty acid, and an iso fatty acid (IFA) is compared to an anteiso fatty acid.

(1) SFA:s and IFA:s occupy smaller areas in a monolayer at the water/air interface (55,89).

(2) SFA:s and IFA:s have higher melting points (79,80,90).

(3) Synthetic phosphatidylcholines containing SFA:s and IFA:s have higher gel to liquid crystalline transition temperatures (91-94).

(4) SFA:s and IFA:s are preferentially incorporated into position 1 of glycerolipids (95,96).

(5) The transition of PE from a lamellar to a non-lamellar phase occurs at a higher temperature when the lipid contains SFA:s and IFA:s (V,97).

(6) The molar fraction of SFA:s and IFA:s in biological membrane lipids increases when the growth temperature is increased (I,VI).

(7) Acyl chains of shorter average length are incorporated into membrane lipids when SFA:s and IFA:s are synthesized (VI,98).

(8) Incorporation of larger relative amounts of SFA:s and IFA:s into *A. laidlawii* membrane lipids results in the same qualitative changes in polar head group composition (I,VI).

The items listed above show that changes in the same direction in the ratios saturated/unsaturated acyl chains and iso/anteiso acyl chains cause similar changes in the physical properties of membrane

lipids containing these pairs of acyl chains. However, the difference between iso and anteiso fatty acids is less than the difference between straight-chain saturated and cis-unsaturated fatty acids. If fatty acids of the same chain length are considered, the four types of acids can be arranged in the order: straight-chain saturated, iso, anteiso, straight-chain cis-unsaturated fatty acids. Monolayer areas increase in the named order; melting points, gel to liquid crystalline transition temperatures of phosphatidylcholines, affinities for the 1-position of glycerolipids, and MGDG/DGDG ratios in the *A. laidlawii* membrane decrease in the named order.

A qualitative application of the self-assembly theory (12,42) can be used to describe the phase equilibria observed in different membrane lipid-water mixtures. The results obtained with these mixtures are highly relevant to the lipid regulation mechanisms observed in *A. laidlawii* and *B. megaterium*. Changes resulting in increased tendencies to form non-lamellar lipid phases produce cell responses that counteract these tendencies and vice versa. Since the membrane lipid compositions are always adapted to new situations, it seems to be the purpose of the regulation mechanisms to maintain a packing of the lipid molecules that results in optimal stability of the bilayer matrix (I,VI). A connection exists between the polar head group composition and the acyl chain composition of the *A. laidlawii* membrane lipids, both when the organism is grown on straight-chain saturated and cis-unsaturated fatty acids (I), and on branched-chain fatty acids (VI). Alterations in the incorporated ratios of palmitoyl/oleoyl chains and iso/anteiso acyl chains yield the same qualitative response in polar head group composition. Changes in the structure of the hydrophobic region, brought about in two different ways but qualitatively

similar (see page 11), thus result in directly correlated changes in the polar head group structure. Moreover, the ratios palmitoyl/oleoyl chains and iso/anteiso acyl chains of the membrane lipids are altered in the same direction in response to changes in the growth temperature. These findings, together with investigations of the phase equilibria in membrane lipid-water systems, suggest that the *effective shape* of the lipid molecules is an important factor governing the regulation of membrane lipid composition in *A. laidlawii* and *B. megaterium* (I,V,VI,13). The effects on lipid composition achieved by incorporation of cholesterol (I) and diethyl ether (99) into *A. laidlawii* membranes also support this statement. Diethyl ether permeates into the hydrophobic region of the lipid bilayer, and should therefore increase the hydrophobic volume (v). As expected, the cells respond by reducing the ratio MGDG/DGDG (99). Results obtained from investigations of the lipid regulation mechanisms occurring in several other biological membranes, can also be convincingly interpreted in terms of geometric and packing properties of the individual lipid species (87). It should however be noted that some results cannot be explained by lipid packing properties. Dioleoyl phosphatidylcholine and the monoglyceride 1-monoolein both form a lamellar phase at room temperature and at a low water content, but an equimolar mixture of these lipids forms a reversed hexagonal phase under the same conditions (100). This indicates that specific interactions between the two lipids are involved.

Many changes in the *A. laidlawii* A membrane lipid composition *cannot* be interpreted as a regulation of the gel to liquid crystalline transition temperature interval; this quantity is often regarded as a measure of the "membrane fluidity" (28,33,101). A strict correlation

between the MGDG/DGDG ratio and the transition temperature interval does not exist. When higher ratios of palmitoyl/oleoyl chains are incorporated into the membrane lipids, the MGDG/DGDG ratio (I) and the transition temperature (47,54) both increase. However, growth of the organism on linolenic acid results in a higher glucolipid ratio than growth on linoleic acid (Å. Wieslander & A. Christiansson, unpublished results), although the former fatty acid results in the lowest transition temperature. This inverse realtionship between the MGDG/DGDG ratio (VI,35) and the transition temperature (88) is exhibited also with elaidic acid and anteiso-C_{15} or anteiso-C_{17}. From investigations of the membrane lipid composition in the related B strain of *A. laidlawii*, it was likewise concluded that there is no general correlation between the gel to liquid crystalline transition temperature and the ratio MGDG/DGDG (88). A regulation of the polar head group composition in *A. laidlawii* A membranes is also obtained when the lipids are totally enriched with *one* acyl chain. When the cells are grown on oleic acid the MGDG/DGDG ratio and/or the amount of ionic lipids are altered as a response to the growth temperature and to cholesterol incorporation (I,64). The gel to liquid crystalline transition temperature differs maximally 15°C between the different *A. laidlawii* polar membrane lipids, and the transition temperature intervals are between 10 and 20°C for acyl chain homogeneous lipids (47,88, 102). A regulation of the transition temperature interval thus seems unlikely.

Lipid compositions resulting in non-lamellar phases of the bulk membrane lipids are actively avoided by the *A. laidlawii* and *B. megaterium* cells (II,III,V). However, most biological membranes contain at least one major lipid species forming a non-lamellar phase (13), and the

synthesis of MGDG never ceases in *A. laidlawii* although the amount of this lipid is powerfully regulated. It is therefore likely that the lipids forming non-lamellar phases fulfil some important functions in a biomembrane. About fifteen years ago it was speculated that lipid phase transitions could be involved in permeation of water through membranes, and in the transmission of receptor signals (18). Somewhat later it was suggested that a temporary and local formation of micelles can play an important role in fusion between cell membranes (103). Some recent experiments can be interpreted to indicate that non-lamellar lipid phases participate in membrane-mediated cell processes such as fusion, exocytosis, and transbilayer transport of lipids and ions (104). Quite recently it was suggested that the contact zones between epithelial cells (tight junctions) consist of pairs of water rods embedded *in* the bilayer structure, and running parallel to the membrane surface (105). It is also possible that lipids with specific molecular geometries are required in order to minimize packing defects around the irregular surfaces of integral membrane proteins (22). The evidences for the occurrence of non-lamellar lipid phases in connection with the above mentioned membrane-mediated processes are still indirect. New methods have to be developed in order to be able to directly observe the presence of non-lamellar phases in biological membranes.

CONCLUSIONS

The regulation of membrane lipid composition in the bacteria *Acholeplasma laidlawii* and *Bacillus megaterium* seems to be governed by the packing properties and the effective shape of the individual

lipid molecules. Changes in membrane lipid composition occurring as a response to variations of the growth conditions should thus be discussed in more specific terms than just a regulation of the gel to liquid crystalline transition temperature, or a regulation of "membrane fluidity". The aims of the lipid regulation mechanisms are more comprehensively understood if several well-defined physico-chemical quantities of the membranes are investigated, e.g. phase equilibria, order parameters, diffusion coefficients, intramolecular motions, permeability, surface charge density and surface potential. A first step towards this approach has been taken in the investigations summarized in this thesis.

In order to obtain a more complete picture of a biological membrane, the membrane proteins must of course be included, and lipid-protein interactions must be studied. The impact of the membrane proteins on lipid packing properties is at present impossible to predict, since these molecules have been shown to both induce and supress the formation of non-lamellar lipid phases (106-108). The relative amount of some *A. laidlawii* membrane proteins is influenced by incorporation of different fatty acids into the membrane lipids (109). The maintenance of a functioning biological membrane under various growth conditions thus requires subtle and interwoven controls of the fatty acid, lipid, and protein syntheses. Studies of these control mechanisms will most certainly be the subject of a large number of future investigations of biological membranes.

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Lipid Bilayer Stability in Membranes. Regulation of Lipid Composition in *Acholeplasma laidlawii* As Governed by Molecular Shape†

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ABSTRACT: The polar lipid composition in membranes of *Acholeplasma laidlawii* is extensively regulated as a response to environmental changes. In particular, the ratio between the dominating lipids monoglucosyldiglyceride and diglucosyldiglyceride is altered depending on temperature, configuration of incorporated fatty acids, and membrane cholesterol content. Synthesis of monoglucosyldiglyceride is stimulated by low temperature and saturated fatty acids but diminished by the presence of cholesterol. These factors are likely to affect the molecular geometry of the membrane lipids. Monoglucosyldiglyceride and diglucosyldiglyceride have wedge- and rodlike molecular shapes, respectively, that are modifiable to a certain extent. The packing constraints of lipids in amphiphilic aggregates, i.e., hydrocarbon–water interfacial area, hydrocarbon chain volume, and hydrocarbon chain length, are very important in determining the aggregate structure [Israelachvili, J. N., Mitchell, D. J., & Ninham, B. W. (1976) *J. Chem. Soc., Faraday Trans. 2 72*, 1525]. Pure monoglucosyldiglyceride forms a reversed hexagonal (H_{II}) phase structure with different fatty acid contents, while diglucosyldiglyceride forms a lamellar phase. However, the only lipid structure compatible with a functional biological membrane is the lamellar phase. Consequently, the balance between lipids forming lamellar and other mesophase structures must keep within certain limits. Here we show that the response in *A. laidlawii* lipid metabolism following external and internal stimuli can be predicted on the basis of molecular shapes and is necessary for the cell in order to maintain optimal membrane stability. Furthermore, the reduced capacity of *Acholeplasma* membranes to incorporate cholesterol is another consequence of this regulation, aiming at preservation of bilayer stability.

The functional state of biological membranes involves a dynamic cooperation between the physiologically active proteins and the lipid matrix. Most physicochemical investigations of these membranes have focused on the gel to liquid crystalline phase transition and its influence on lipid lateral packing (Engelman, 1971), growth temperature adaptation (McElhaney, 1974), permeability characteristics of water, ions, and other species (Razin, 1975a), enzyme and transport activities (Read & McElhaney, 1975; Silvius et al., 1978), and protein disposition (Verkleij, 1975).

Concerning the contribution of lipid polar head groups to membrane function and stability, little is known. However, the maintenance of a critical balance between lipids with different anionic polar head groups has been observed in *Escherichia coli* (Raetz, 1978). Recent findings also show that the activities of a very limited number of membrane-associated enzymes are dependent on a specific lipid surrounding (Sandermann, 1978).

Our previous investigations have focused on the relationships between physiological regulation mechanisms and physical

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properties of membrane lipids from the wall-less procaryote *Acholeplasma laidlawii* strain A (Carlemalm & Wieslander, 1975; Wieslander & Rilfors, 1977; Christiansson & Wieslander, 1978, 1980; Wieslander et al., 1978, 1979). Three distinct ways for regulating the membrane lipid composition have been discovered in this strain: (1) incorporation of fatty acids into membrane lipids is governed by growth temperature as well as the presence of cholesterol; (2) the relative syntheses of the dominating polar lipids monoglucosyldiglyceride (MGDG)[1] and diglucosyldiglyceride (DGDG) are related to each other; they are influenced by changes in the apparent molecular ordering of the hydrocarbon chains caused by growth temperature, fatty acid content, and the presence of cholesterol; (3) the total balance between ionic and nonionic lipids seems to depend on the average lateral packing area occupied by the lipid molecules in the membrane. Such drastic changes in polar head group composition and in the ratio ionic/nonionic lipids have not been observed in other membranes, cf. *E. coli* wild type (Raetz, 1978). Investigations on lipid packing properties in the dry crystalline state (Carlemalm & Wieslander, 1975; Carlemalm, 1976), water binding capacities, transition temperatures, phase structures (Wieslander et al., 1978), and lateral diffusion (Å. Wieslander, L. Rilfors, L. B.-Å. Johansson, and G. Lindblom, unpublished results) of membrane lipids from this *A. laidlawii* strain have also been performed.

In this paper we will show how the physical properties of the membrane lipids of *A. laidlawii* can be related to membrane stability. The only lipid structure compatible with a functional biological membrane is the lamellar one. However, it has been found that often this phase is not the thermodynamically stable one formed by a single membrane lipid–water system at physiological temperatures. Typical membrane lipids forming hexagonal mesophases are phosphatidylethanolamine (PE) (Cullis & De Kruijff, 1978b), diphosphatidylglycerol (DPG) (Rand & Sengupta, 1972), monogalactosyldiglyceride (Shipley, 1973), MGDG (Wieslander et al., 1978), and sphingomyelin (Yeagle et al., 1978). Mixtures of MGDG and DGDG can form a cubic mesophase (Å. Wieslander, L. Rilfors, L. B.-Å. Johansson, and G. Lindblom, unpublished results).

The tendencies for amphiphiles to form different structures (micelles and lamellar or hexagonal phases) can be understood from a unified theory that links interaction free energy, molecular geometry, and thermodynamics (Israelachvili et al., 1976, 1977). Two opposing forces contribute to the interaction free energy. The attractive interactions are the hydrophobic effect (Tanford, 1973), which tends to eliminate the contact between water and the hydrophobic core. Repulsive interactions arise from electrostatic and steric head group repulsion. Steric acyl chain repulsion is also involved. These interactions aim at keeping the total interaction free energy per lipid molecule at a minimum, resulting in an optimal surface area per head group, i.e., the area occupied by the polar head at the hydrocarbon–water interface. Thus, the packing constraints of the lipids in the amphiphilic aggregate are important factors in determining the aggregate shape (Israelachvili et al., 1976, 1977). These authors derived geometric expressions relating the hydrocarbon–water interfacial area a, the hydrocarbon chain volume v, and the hydrocarbon chain length l to the aggregate structure. The following results were obtained for various aggregate shapes: spherical micelle, $v/al = 1/3$; rodlike micelle, $v/al = 1/2$; lamellae, $v/al = 1$. It follows that v/al must be larger than 1 for an aggregate building up a reversed hexagonal (H_{II}) phase, i.e., water cylinders in a hydrocarbon matrix (Ekwall, 1975).

Recently, Wennerström (1979) showed that the theory by Israelachvili et al. is of great importance for the understanding of the phase diagrams of soap–water systems. He concludes that the formation of a lamellar phase on addition of alcohol to a micellar solution or a hexagonal (H_I) phase is due to the fact that the hydrocarbon volume v is enhanced, i.e., v/al approaches unity. Similarly, the lipid organization in a bilayer is a delicate balance between various packing constraints, which determine the membrane stability. All other mesophase structures but the lamellar phase are prohibited. Thus, in a multicomponent bilayer system the geometrical properties of the individual lipid molecules are very important factors. In this work we apply the self-assembly theory by Israelachvili et al. to explain the observed physiological regulation of the different major lipids in *A. laidlawii*. It is found that the response in lipid metabolism following external and internal stimuli can be predicted by the theory.

Results and Discussion

Synthesis and Physical Properties of Membrane Lipids. A. laidlawii has an obligate demand for unsaturated fatty acids that it cannot synthesize (Pollack & Tourtellotte, 1967). Saturated fatty acids are synthesized by the malonyl-coenzyme A pathway (Rottem & Panos, 1970). This synthesis is inhibited by exhaustion of pantethine, a precursor to coenzyme A and acyl carrier protein, from the growth medium (Christiansson & Wieslander, 1980). An absolute control of fatty acid incorporation from exogenous sources can thus be achieved (Wieslander & Rilfors, 1977; Christiansson & Wieslander, 1978, 1980).

In *A. laidlawii* strain A (EF22) the following polar lipids occur (Wieslander & Rilfors, 1977): MGDG, DGDG, an apolar monoglucolipid, phosphatidylglycerol (PG), and glycerophosphoryl derivatives of MGDG and DGDG. Under certain growth conditions DPG is synthesized (Christiansson & Wieslander, 1978). Although uncharged at physiological pH, the glucolipids are cationic selective (Hopper et al., 1970) and calcium ion binding (Wieslander et al., 1978) in resemblance to PE. PG, DPG, and the phosphoglucolipids each carry one negative charge. The tentative lipid biosynthetic pathways are shown in Figure 1.

^{2}H NMR on heavy water and ^{2}H-labeled acyl chains together with low-angle X-ray diffraction for lipid–water samples reveal a reversed hexagonal (H_{II}) phase structure for MGDG with different fatty acid contents, whereas DGDG and the other lipids form lamellar phases (Wieslander et al., 1978). DPG can alter between the two phase structures (Rand & Sengupta, 1972). Maximum hydration (7–9 mol of H_2O/polar head group) and phase transition temperatures for MGDG and DGDG are very similar to those of both synthetic and naturally occurring PE (Wieslander et al., 1978). The transition temperatures are about 20 °C above that of phosphatidylcholine with the corresponding fatty acid content (Wieslander et al., 1978).

Lipid Molecular Shape. The various lipid molecules in the bilayer can be visualized as building blocks with different geometries (Figure 2). The hydrocarbon chain length, hydrophobic volume, and polar head group area will determine the overall shape of the blocks (see also introductory statement). The phase structure formed by the different lipids is determined by this molecular shape.

[1] Abbreviations used: MGDG, monoglucosyldiglyceride; DGDG, diglucosyldiglyceride; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; NMR, nuclear magnetic resonance.

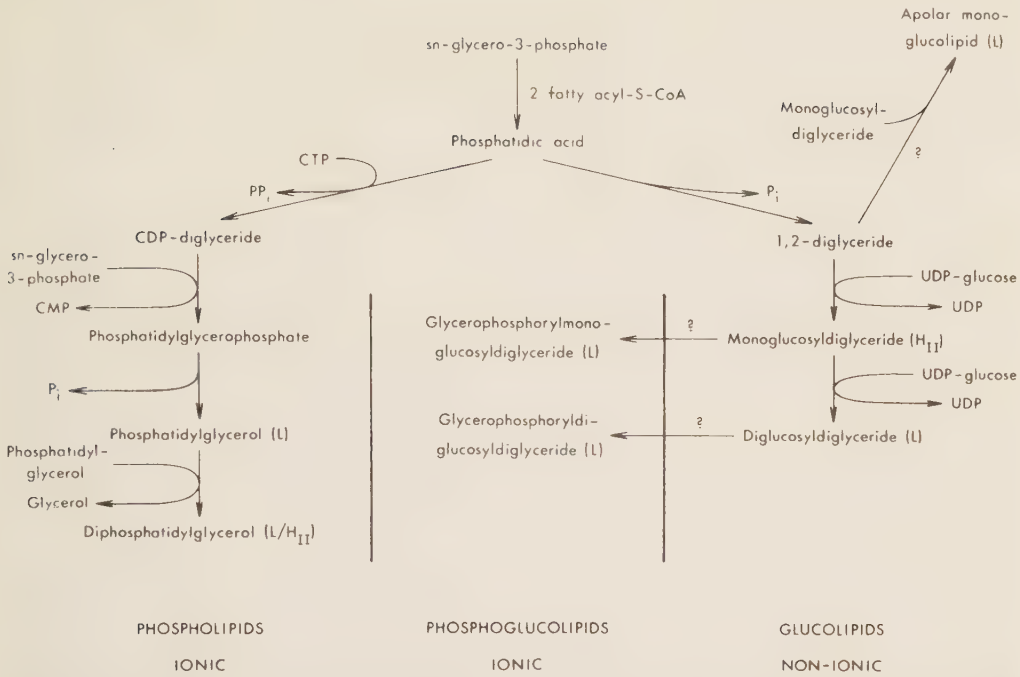

FIGURE 1: Tentative pathways for membrane polar lipid biosynthesis in *Acholeplasma laidlawii* strain A. Abbreviations used: CMP, cytidine 5′-monophosphate; CDP, cytidine 5′-diphosphate; CTP, cytidine 5′-triphosphate; UDP, uridine 5′-diphosphate; P_i, inorganic orthophosphate; PP_i, inorganic pyrophosphate; CoA, coenzyme A; L, lamellar phase structure; H_{II}, reversed hexagonal phase structure; ?, suggested pathway.

(i) With a given *fatty acid composition*, i.e., constant values of v and l, any change in the size (bulkiness, charge, hydration) of the polar head group will affect the optimal surface area a of the lipid molecule. Modifications of polar head structure can thus alter the packing properties (v/al) of an individual lipid in the bilayer (Figure 2A).

(ii) For lipids with a given *polar head group*, i.e., constant value of a, the molecular shape depends on several factors. A substitution of unsaturated fatty acids (Figure 2B, left) for saturated ones, especially with cis double bonds (Figure 2B, right), will reduce the length of the hydrocarbon chains (l) and increase the width of the hydrocarbon space almost without any change in the hydrophobic volume (v) (Träuble & Haynes, 1971; Israelachvili et al., 1977). By increasing the temperature, trans–gauche isomerizations and 2g1 kinks are introduced into the hydrocarbon chains (Mendelsohn & Taraschi, 1978; Träuble & Haynes, 1971), yielding the same effects as mentioned above (Figure 2B).

(iii) *Cholesterol* has a pronounced wedge shape (Carnie et al., 1979; Figure 2B). Furthermore, cholesterol slightly diminishes the lateral packing area of an individual lipid in a monolayer, since the observed mean molecular areas for cholesterol–lipid mixtures are smaller than those obtained by theoretical calculations (Demel & De Kruijff, 1976). However, the presence of cholesterol will nevertheless largely increase the distances between all the other lipid molecules.

Lipid Regulating Mechanisms in Acholeplasma laidlawii. (*A*) *Fatty Acid Incorporation.* The potential capacity of *A. laidlawii* to regulate membrane fatty acid incorporation is best evoked by applying a temperature-shift technique (Christiansson & Wieslander, 1978). Cells grown in an equimolar mixture of palmitic and oleic acid incorporate more oleic acid when shifted to 17 °C than cells maintained at 37 °C. This increase of oleic acid takes different courses with respect to different lipid species and time after the temperature decrease. The most rapid response occurs in MGDG, the polar lipid having the highest content of palmitic acid. In PG the oleic acid content rises after an initial lag period, whereas DGDG remains nearly unchanged. Thus, in order to maintain an optimal packing of the various lipids in the bilayer, according to the self-assembly theory, the cell can "afford" to incorporate unsaturated fatty acids into MGDG, although this lipid forms a reversed hexagonal (H_{II}) phase. In fact, since the temperature is lowered, it can be expected that the MGDG content is responsible for about the same packing constraint at the low temperature as it was at the higher temperature with more saturated fatty acids (Figure 2B), giving an optimal packing in the bilayer.

(*B*) *Regulation of Polar Head Group Composition.* Three different ways to change the polar head group composition have been exploited in *A. laidlawii* strain A (Wieslander & Rilfors, 1977; Christiansson & Wieslander, 1978, 1980): (i) variations in structure of incorporated *fatty acids*; (ii) changes in growth *temperature*; (iii) incorporation of *cholesterol*. The predominant response in polar lipid metabolism is a large change in the ratio between the structurally related glucolipids MGDG and DGDG (Figure 1). Furthermore, the ionic lipid fraction varies profoundly. These changes in *A. laidlawii* play a central role concerning the relation between lipid molecular shape and membrane bilayer stability.

(i) Successive changes in the hydrophobic geometry of the lipids caused by different incorporated ratios of saturated/unsaturated *fatty acids* yield correlated responses in the molar ratio MGDG/DGDG (Table I). Increased amounts of cis-

Table I: Polar Lipid Composition in Membranes from *A. laidlawii* A Grown with Different Amounts of Saturated and Unsaturated Fatty Acids (18 h, 37 °C)

	supplementation to the growth medium (μM palmitic/μM oleic acid)					
	120/30	90/60	75/75	30/120	0/150	0/150 + 20 μM cholesterol[a]
% oleic acid in lipids (mol/mol)	30.3	44.4	49.6	55.2	95	95
ratio MGDG/DGDG	2.37	1.21	0.80	0.75	0.57	0.27
% ionic lipids of total (mol/mol)	13.5	17.2	22.9	25.5	35.9	43.1

[a] The ratio cholesterol/total lipids is 0.29 (mol/mol), corresponding to about 15% (wt/wt), which is the maximum incorporable amount in *A. laidlawii*.

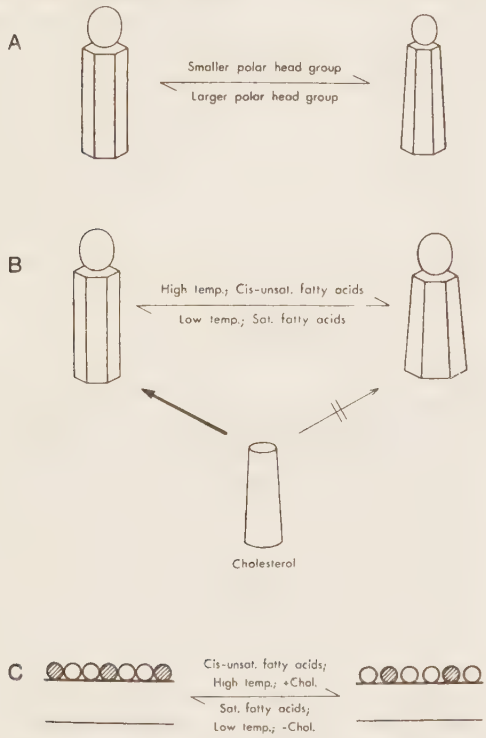

FIGURE 2: Molecular shapes of lipids. The geometrical properties of the lipid molecules determine their ability to pack together into a bilayer structure. (A) Molecules with identical fatty acid compositions. Left: geometry of lipids having a moderately large polar head group and a tendency to form a lamellar phase structure. Right: geometry of lipids having a small polar head group and thus a tendency to form a reversed hexagonal (H_{II}) phase structure due to the reduced hydrocarbon–water interfacial area. Note the pronounced wedge shape of the molecule. (B) Molecules with identical polar head groups. Left: geometry of lipids at temperatures below the phase transition temperature (T_t) and/or with saturated fatty acids only. Right: geometry of lipids at temperatures above T_t and/or containing cis unsaturated fatty acids. The shortening and broadening of the hydrophobic part of the molecule result in an increased tendency to form a reversed hexagonal (H_{II}) phase structure. Lower middle: molecular geometry of cholesterol. The arrows denote the possibilities for cholesterol to copack with lipids having different geometries and still preserve a stable bilayer structure. The dimensions of the molecules are based on literature values. (C) Membrane surface charge density. Left: polar head group density at temperatures below T_t and/or with lipids containing saturated fatty acids only. Right: polar head group density at temperatures above T_t and/or with lipids containing cis unsaturated fatty acids and/or cholesterol. Hatched circles denote anionic lipid molecules.

unsaturated fatty acids will increase the hydrophobic bulkiness of the lipid molecules (Figure 2B), leading to a more accentuated wedge shape, especially of MGDG, which fits better in a curved aggregate than in a bilayer structure. Consequently, the membrane stability is maintained by *decreasing* the ratio MGDG/DGDG upon an increase of cis unsaturation. The extent of variation of MGDG is 60% larger than that of DGDG, indicating that MGDG is the most actively regulated component. Similar alterations in the ratio MGDG/DGDG occur when the endogenous saturated fatty acid synthesis is stimulated by stepwise additions of the coenzyme precursor pantetheine (Christiansson & Wieslander, 1980).

By decreasing the molar fraction of cis-unsaturated fatty acid in the membrane from 95 to 30%, the ionic lipid fraction is reduced by almost a factor of three (Table I). This might be due partly to a compensation for the enhanced surface charge density caused by the decreased lateral packing area of the lipid (Figure 2C). Since the presence of divalent cations in the culture medium raises the gel to liquid crystalline transition temperature for the ionic lipids (Träuble, 1977), a reduction in the amount of these lipids may also be necessary to prevent the molecular ordering of the acyl chains from becoming too high.

(ii) A decrease in *temperature* leads to an increase in molecular order of the acyl chains, diminishing the hydrophobic bulkiness of the lipids (Figure 2). The magnitude of this effect depends on the existing fatty acid composition before the temperature shift (Figure 2B). Most biological membranes contain a certain fraction of wedge-shaped polar lipids. The presence of these lipids is of importance for bilayer stability, and, consequently, a decrease in temperature must be neutralized by accentuating the wedge shape properties. This can be achieved in two ways: (1) an enhanced incorporation of unsaturated fatty acids (section i above) and (2) increased synthesis of lipids with a small polar head group like MGDG (Figure 2A). The latter effect is demonstrated in Figure 3, where it is shown how the ratio MGDG/DGDG is altered with cell growth after a temperature shift and/or with different membrane fatty acid compositions. At the time of shift-down (12 h), the ratio of MGDG/DGDG differs significantly depending on the difference in fatty acid composition as shown in Table I. With both fatty acid supplementations this ratio is diminished during growth at 37 °C (Wieslander & Rilfors, 1977; Christiansson & Wieslander, 1978). However, at 17 °C the ratio rises due to a largely *enhanced* synthesis of MGDG (Christiansson & Wieslander, 1978). This effect is observed also when *A. laidlawii* is grown at a constant low temperature, 10 °C. Because of the shape of the molecule, MGDG will reestablish an optimal packing of the membrane components in the bilayer.

When passing the phase transition interval a decrease in temperature will decrease the lateral packing area of the lipids

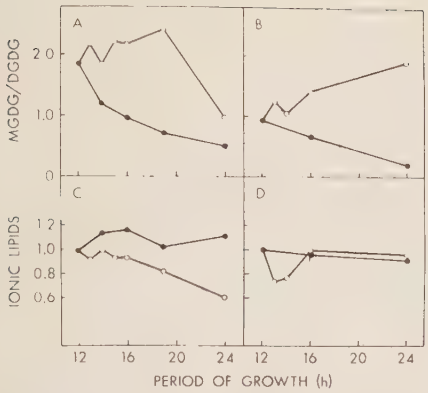

FIGURE 3: Regulation of polar lipid composition in *A. laidlawii* after a temperature shift: (A and B) ratio MGDG(H_{II})/DGDG(L); (C and D) relative amount of ionic lipids; the amount at 12 h is assigned the value of 1.0; (A, C) cells grown with 75 μM palmitic acid and 75 μM oleic acid; (B, D) cells grown with 150 μM oleic acid; (●) growth at 37 °C; (○) cells shifted to 17 °C after growth for 12 h at 37 °C. The experimental conditions are described by Christiansson & Wieslander (1978).

and consequently increase the surface charge density (Träuble, 1977; Träuble & Haynes, 1971; Figure 2C). With membranes containing palmitic and oleic acid, a shift from 37 °C to a temperature within the phase-transition interval, e.g., 17 °C (Christiansson & Wieslander, 1978; Wieslander et al., 1978) yields a strong reduction in the biosynthesis of ionic lipids (Figure 3C). The reasons for this may be the same as mentioned in section i. With membranes containing oleic acid only, 17 °C is well above the phase-transition interval (cf. Figures 3C and 3D).

(iii) Addition of *cholesterol* to an *A. laidlawii* culture grown with an equimolar mixture of palmitic and oleic acid yields higher incorporation of oleic acid both at 37 °C and after a shift to 17 °C (Christiansson & Wieslander, 1978). Obviously cholesterol causes an increase in the ordering of the fatty acyl chains resulting in a more rodlike type of molecular geometry of the surrounding lipids. This is counteracted by an increased incorporation of unsaturated fatty acids in all lipids. Furthermore, since cholesterol has a significant wedge shape (Carnie et al., 1979; Figure 2B), insertion of this molecule into the membrane destabilizes the bilayer structure, especially in the presence of lipids forming a reversed hexagonal (H_{II}) phase. In *A. laidlawii* the formation of such a phase in the membrane is prevented by a relative *reduction* of the amount of MGDG by 40% (Table I). Incorporation of cholesterol into a membrane also dilutes the surface charge density (Figure 2C). *A. laidlawii* compensates for this by increasing the ionic lipid fraction (Table I).

Thus, according to the presented theory, an increase in the number of cis double bonds, an increase in temperature, as well as the presence of cholesterol destabilize the lamellar phase structure, which the cell must compensate for. This indeed occurs by a change in the polar head composition: a lowering of the MGDG lipid content and an increase in the amount of ionic lipids.

Concluding Remarks

It has been shown that for most purposes a qualitative application of the theory developed by Israelachvili et al. (1976, 1977) can be used for a sufficiently good description of the lipid bilayer stability in *Acholeplasma laidlawii* membranes. Thus, it can be concluded that the molecular geometry of the lipids is of vital importance for the stability. Although the theory has been applied to the membrane of *A. laidlawii*, it can nevertheless be expected to hold also for other biological membranes. For example, it has been found that *E. coli* counteracts the effect of a drop in the growth temperature by an increase in the degree of cis unsaturation in the lipids (Cronan & Gelmann, 1975), which changes the geometry of the dominating polar lipid PE to a more pronounced wedge shape (Cullis & De Kruijff, 1978a). In *Bacillus megaterium* this temperature adaptation is accomplished by regulation of the fatty acid chain length and by the ratio between two different types of branched fatty acids (Rilfors et al., 1978), which influence the hydrophobic volume.

A proposal can be made to explain the occurrence of several different polar lipid species in the membranes of most organisms, and why their relative amounts must be actively regulated. These membrane properties most certainly would have been lost during evolution were they not of crucial importance for survival to the cell. An optimal packing of the lipid molecules is essential for obtaining a stable bilayer structure. This demand cannot be fulfilled by a single lipid species; it necessitates a mixture of lipids having tendencies to form lamellar and other mesophase structures (e.g., hexagonal, cubic). As shown in this work, the balance between such lipids must keep within certain limits, and this probably explains why some lipid species are not coexisting in biological membranes (Demel et al., 1977; Carnie et al., 1979). Furthermore, it has been proposed that local regions of lipids forming transitory nonlamellar phases may be advantageous to some membrane functions such as movement of membrane components between bilayer halves, fusion, exo- and endocytosis, and facilitated transport (Cullis & Verkleij, 1979). Nonlamellar phases have been reported for microsomal membranes (De Kruijff et al., 1978; Stier et al., 1978). It has also been suggested that packing constraints impose a structural coupling between membrane proteins and their surrounding lipids, and thus a heterogeneous composition of lipids with different molecular geometries may be required in order to minimize packing defects around the irregular surfaces of proteins (Israelachvili, 1977; Sandermann, 1978). This suggestion is strongly supported by protein–lipid interaction studies performed on *A. laidlawii* (K.-E. Johansson, C. Jägersten, A. Christiansson, and Å. Wieslander, unpublished experiments).

The balance between ionic and nonionic lipids is actively regulated in *A. laidlawii* membranes (Table I). The great capability of *A. laidlawii*, compared to *E. coli*, to regulate the ionic lipid fraction in response to environmental changes seems logical considering that the *A. laidlawii* membrane directly faces the surrounding physical milieu, while the *E. coli* membrane is enclosed by a cell envelope.

Our results also show the significance of knowing the phase diagram of the individual as well as mixed compositions of the various membrane lipids. Hitherto, few such diagrams have been reported in the literature. Recently, we discovered a cubic phase consisting of a mixture of MGDG and DGDG (Å. Wieslander, L. Rilfors, L. B.-Å. Johansson, & G. Lindblom, unpublished experiments). This phase is formed by introducing surplus amounts of MGDG with pronounced wedge shape (i.e., containing cis unsaturated fatty acids) in the lamellar phase of DGDG. The observation further supports the self-assembly theory used.

It should be noted that the theory also nicely pertains to the effect of cholesterol on biological membranes. Monosugar diglycerides, forming a reversed hexagonal (H_{II}) phase

structure, are present in significant amounts in the membranes of all *Acholeplasma* species (Smith, 1979). The genus *Acholeplasma* is also characterized by having no requirement for cholesterol (Razin, 1978). Moreover, in resemblance to cell wall enclosed bacteria, *A. laidlawii* has a reduced capacity for cholesterol incorporation (Razin, 1975b). The presence of this molecule in the membrane diminishes the MGDG content (Table I). In light of the observed phase behavior of MGDG/DGDG mixtures (see above), it can be anticipated that large amounts of the wedge-shaped cholesterol molecule must induce a nonlamellar phase just as MGDG does. Hence, the *simultaneous* presence of large amounts of MGDG and cholesterol must be avoided. This finding is supported by the observation that cholesterol destabilizes the bilayer structure of dioleoylphosphatidylethanolamine as well as a mixture of soy phosphatidylethanolamine and egg yolk phosphatidylcholine (Cullis & De Kruijff, 1978a). Previously, the results obtained usually were discussed in terms of cholesterol as a moderator of "fluidity" or "microviscosity". This explanation has recently been questioned (G. Lindblom, L. B.-Å. Johansson, & G. Arvidson, unpublished experiments), since it was observed that the lipid lateral diffusion (a *dynamic* parameter) was practically unaffected by the presence of cholesterol in the bilayer. We propose that cholesterol, due to its molecular shape, exerts its greatest influence on bilayer structures by disturbing the packing of the lipid molecules.

Acknowledgments

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The Effect of Cholesterol on the Phase Structure of Glucolipids from *Acholeplasma laidlawii* Membranes

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1. The packing properties in lipid mixtures containing cholesterol and membrane glucolipids from *Acholeplasma laidlawii* are modified by varying the amounts of cholesterol, diacylmonoglucosylglycerol and diacyldiglucosylglycerol in the mixtures as well as the temperature and the degree of acyl chain unsaturation. These changes affect both the proportions of different lipids having dissimilar molecular geometries and the geometry of the lipid molecules themselves.

2. All mixtures containing glucolipids with equal amounts of palmitoyl and oleoyl chains formed a lamellar liquid-crystalline phase in the growth temperature range of *A. laidlawii*, while a reversed cubic liquid-crystalline phase dominated in mixtures containing dioleoyl glucolipids. These lipids formed a reversed hexagonal phase together with 27% cholesterol. Mixtures with lipid compositions occurring in the membranes of living *Acholeplasma* cells formed a lamellar liquid-crystalline phase.

3. Large amounts of cholesterol and diacylmonoglucosylglycerol, high temperatures and a high degree of *cis* unsaturation favoured the formation of cubic or hexagonal liquid-crystalline phase structures of the investigated lipid mixtures. Diacylmonoglucosylglycerol and cholesterol are both wedge-shaped. Temperature and *cis* unsaturation accentuate the wedge-shape properties of the glucolipid molecules.

4. The changes in the lipid composition of *A. laidlawii* membranes as a response to cholesterol incorporation can be explained by the geometry and packing characteristics of the sterol molecule and the concept of 'fluidity' does not need to be involved.

The organization of membrane components is governed by energetic, entropic and steric constraints, and the thermodynamically possible ways to pack the components together are limited by these constraints. The geometry of amphiphiles is of particular importance for the formation of lipid aggregates with different shapes [1]. The lamellar phase is a prerequisite for obtaining a stable, insulating biological membrane. However, most of such membranes contain significant amounts of at least one lipid species which, owing to its molecular shape, cannot form a lamellar phase. It has been proposed that local regions of lipids forming non-lamellar structures may be advantageous to certain membrane functions [1]. Diacylmonoglucosylglycerol ($Acyl_2GlcGro$) forms a reversed hexagonal (H_{II}) phase with different acyl chain contents [2], while phosphatidylethanolamine exhibits a lamellar or an H_{II} phase depending on temperature, acyl chain content and pH [3]. $Acyl_2GlcGro$ and unsaturated phosphatidylethanolamine can be pictured as truncated cones. Cholesterol is a flat, rigid molecule compared to lipids containing flexible chains and does not form bilayers at physiological temperatures [1]. Cholesterol has been observed to destabilize the bilayer structure of dioleoylglycerophosphoethanolamine as well as a mixture of soya phosphatidylethanolamine and egg-yolk phosphatidylcholine [4]. Large amounts of phosphatidylethanolamine and cholesterol are usually not found together in biological membranes cf. [1,5].

Cholesterol is present in most membranes of eucaryotic cells [5]. Procaryotes, except for the mycoplasmas, are devoid of this lipid. The *Mycoplasma* species have a strict demand for cholesterol, and can incorporate up to 50 mol/100 mol of this lipid in their membranes [6]. However, within the mycoplasma group, the genus *Acholeplasma* lacks the growth requirement for cholesterol. It has, nevertheless, a reduced capacity for cholesterol incorporation, similar to that of cell-wall-enclosed bacteria [7]. Living *Acholeplasma laidlawii* cells have a control mechanism limiting the amount of cholesterol incorporated into their membranes, although the uptake itself is not an active physiological process [8]. The mechanism responsible for the difference in cholesterol incorporation into membranes of *Mycoplasma* and *Acholeplasma* species is poorly known [6,9].

The lipid composition in *A. laidlawii* membranes is influenced by growth temperature, acyl chain composition and cholesterol content [10–13]. Temperature and acyl chain composition determine the shape of the lipid molecules [1]. We have recently shown that the regulation of lipid composition is a mechanism whereby *A. laidlawii* always secures an optimal bilayer stability [14]. The regulation is expressed as an alteration in the relative syntheses of the two dominating lipids $Acyl_2GlcGro$ and diacyldiglucosylglycerol ($Acyl_2Glc_2Gro$), the latter lipid forming a lamellar phase [2]. The amount of ionic lipids is also changed [14]. Studies of mixtures of $Acyl_2GlcGro$ and $Acyl_2Glc_2Gro$ *in vitro* showed that a high degree of *cis* unsaturation and large amounts of $Acyl_2GlcGro$, i.e. factors increasing the wedge-shape properties of the lipids,

Abbreviations. $Acyl_2GlcGro$, diacylmonoglucosylglycerol; $Acyl_2Glc_2Gro$, diacyldiglucosylglycerol; Pam, palmitoyl; Ole, oleoyl; PamOleGlcGro, palmitoyloleoylmonoglucosylglycerol; $Ole_2GlcGro$, dioleoylmonoglucosylglycerol; $PamOleGlc_2Gro$, palmitoyloleoyldiglucosylglycerol; Ole_2Glc_2Gro, dioleoyldiglucosylglycerol; Cho, choline; Etn, ethanolamine; H_{II}, reversed-hexagonal liquid crystal; Q_{II}, reversed-cubic liquid crystal.

favour the formation of a reversed cubic (Q_{II}) phase. This phase consists of two mutually interwoven, three-dimensional water rod system in a lipid matrix [15]. It was concluded that the *Acholeplasma* cells actively avoid $Acyl_2GlcGro/Acyl_2$-Glc_2Gro ratios promoting the formation of the cubic phase [15].

In this paper we report the effect of temperature and cholesterol on the phase structure of mixtures of $Acyl_2GlcGro$ and $Acyl_2Glc_2Gro$. The results can be predicted by the theory dealing with self-assembly of amphiphiles presented by Israelachvili et al. [1,16]. It is shown here that Q_{II} or H_{II} phases are formed with increasing temperature and large cholesterol concentrations. The lower amount of cholesterol in *Acholeplasma* membranes as compared to *Mycoplasma* membranes most likely depends on the low solubility of cholesterol in the glucolipids and the instability of the lamellar phase, which is induced by cholesterol in the presence of lipids forming an H_{II} phase such as $Acyl_2GlcGro$.

MATERIALS AND METHODS

Membrane Lipids

Growth of *Acholeplasma laidlawii* A, strain EF22, and preparation of the membrane lipids was performed as described previously [15]. Lipids with two different acyl chain compositions were used: (a) lipids with about equal amounts of palmitoyl and oleoyl chains; (b) lipids with $\geq 95\%$ oleoyl chains (Table 1).

Sample Preparation

Different mixtures of $Acyl_2GlcGro$, $Acyl_2Glc_2Gro$ and cholesterol were prepared by adding the components one at a time to an NMR tube. The lipids were mixed to complete solubility and optical clarity in organic solvents. These were removed completely by drying the samples for 24 h at a pressure less than 0.1 mm Hg (13 Pa). The composition of the mixtures was determined by weighing the NMR tubes plus content. Finally 11% (w/w) of ^2H_2O was added. The samples were flushed with N_2 and the deuterated water was mixed with the lipids by extended centrifugation at 45 °C. The deuterated water concentration used is slightly lower than the maximum hydration capacities of these lipids [2,15]. Water concentrations above these limits were avoided since free water may complicate the interpretation of the NMR spectra. H_{II} and cubic liquid-crystalline phases have been shown to be in equilibrium with dilute aqueous solution phases [2,15]. After NMR analysis, samples were analyzed for lipid degradation by thin-layer and gas/liquid chromatography as described [2].

^2H NMR

The NMR spectrum of deuterated water is dominated by the interaction of the deuteron quadrupole moment with the electric field gradients at the nucleus. For anisotropic liquid-crystalline samples this quadrupole interaction generates a spectrum with two equally intense peaks. In an isotropic solution, on the other hand, this interaction is averaged to zero as a result of the rapid molecular motions, and the spectrum will consist of a sharp singlet line. The observed quadrupole splitting, Δ^2H_{obs}, measured as the distance between the two peaks in frequency units, depends on the fraction of deuterons in one or several anisotropic sites, the quadrupole coupling constant, and the average molecular ordering of water molecules in the sites. A detailed theoretical treatment of quadrupole splittings in lyotropic liquid crystals has been published by several authors [17–21]. It has been shown previously that ^2H NMR of deuterated water can be conveniently used to study phase equilibria of membrane lipids [2,22].

For a heterogeneous system consisting of two or more phases one expects a superposition of ^2H NMR spectra characterizing the phases, provided that the deuteron exchange between them is slow. Thus, for a system containing a mixture of a lamellar and a hexagonal phase, two quadrupole splittings are usually observed. In such a mixture the values of the splittings follow the relation [17]:

$$\Delta^2H_{lamellar} = -{}^1/_2\,\Delta^2H_{hexagonal}$$

provided that (a) the lamellae show no appreciable curvature, (b) the local interactions and structures are the same for the two phases and (c) translational diffusion around cylinders in the hexagonal structure occurs in a time shorter than the inverse splitting. For a three-phase system, consisting of two liquid crystals and one isotropic solution, the ^2H NMR spectrum will yield two doublets and a central singlet.

Experimental Procedure

^2H NMR spectra were studied at 15.351 MHz on a modified Varian XL-100-15 pulsed spectrometer working in the Fourier-transform mode using an external lock. A variable temperature control unit was used to control the temperature of the gas flow in the NMR probe, in which the sample was situated. Pre-cooled (methanol/solid carbon dioxide) N_2 gas was used for low temperatures (−10 – +28 C) and air for high temperatures (30–60 °C). The samples were thermally equilibrated within 1.0 °C for at least 1 h before ^2H NMR spectra were taken. Acquisition time was 0.2 s and number of transients was 20.000–60.000. The temperature of each sample was determined before and after running each experiment using a mercury thermometer. The experiment was repeated for all samples after about one month and no noticable change was detected. In addition to NMR analysis, the samples were also studied by polarizing light microscopy. Different phases have very typical textures that are easily recognized [23].

RESULTS

The effect of cholesterol on the phase structure of the membrane glucolipids from *Acholeplasma laidlawii* was studied as a function of lipid composition. The amount of wedge-shaped lipids in the samples was varied by changing the amount of $Acyl_2GlcGro$ and cholesterol (Table 1). In addition, the molecular shapes of $Acyl_2GlcGro$ and $Acyl_2Glc_2Gro$ were affected by changing the amounts of *cis*-unsaturated acyl chains in the lipids and by varying the temperature. The amounts of cholesterol in the samples, i.e. 27% and 50% (mol/mol), correspond to the maximum incorporable amount in *A. laidlawii* and *Mycoplasma* membranes respectively.

Fig. 1 contains typical NMR spectra from samples with different phase structures. The obtained deuteron quadrupole splittings and the phase structures of the samples at various temperatures have been summarized in Table 2.

Table 1. *Composition of mixtures made from* Acholeplasma laidlawii *membrane glucolipids and cholesterol*
The $Acyl_2GlcGro/Acyl_2Glc_2Gro$ mixture was 50–90 mg together

Sample	Molar ratio $Acyl_2GlcGro/Acyl_2Glc_2Gro$	Cholesterol	Glucolipid species	Acyl chain composition	
				palmitoyl	oleoyl
		mol/100 mol		mol/100 mol	
I	0/1.0	0			
IIa	1.0/1.0	0			
IIb	1.0/1.0	27			
IIc	1.0/1.0	50	$Acyl_2GlcGro$	54	46
IIIa	2.0/1.0	0	$Acyl_2Glc_2Gro$	45	55
IIIb	2.0/1.0	27			
IIIc	2.0/1.0	50			
IV	0/1.0	0			
Va	1.2/1.0	0			
Vb	1.2/1.0	27	$Acyl_2GlcGro$	–	≥ 95
VIa	2.5/1.0	0	$Acyl_2Glc_2Gro$	–	≥ 95
VIb	2.5/1.0	27			

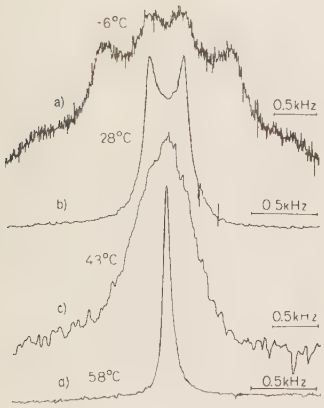

Fig. 1. *Typical ^2H NMR of glucolipid/cholesterol mixtures.* (a) $Ole_2GlcGro/Ole_2Glc_2Gro$ 2.5/1.0 (mol/mol) mixture with 27% (mol/mol total lipids) cholesterol recorded at −6 °C. Two-phase sample containing lamellar and hexagonal phases; (b) the same sample as in (a) recorded at 28 °C. Hexagonal phase; (c) the same sample as in (a) recorded at 43 °C. Hexagonal phase, unresolved quadrupole splitting (d) $Ole_2GlcGro/Ole_2Glc_2Gro$ 1.2/1.0 (mol/mol) mixture recorded at 58 °C. Cubic phase consisting of two mutually interwoven, three-dimensional water rod systems in a lipid matrix, for details see [15]

Palmitoyloleoyl Glucolipids without Cholesterol

The ratio between $Acyl_2GlcGro$ and $Acyl_2Glc_2Gro$ (Table 1) in samples IIa (1/1) and IIIa (2/1), but not in sample I (0/1) occur *in vivo*, but only with this acyl chain composition [10–13]. All palmitoyloleoyl glucolipid mixtures without cholesterol form lamellar phases in the physiological temperature range (approx. 10–43 °C), [2, 15].

Table 2. *Quadrupole splittings for different glucolipid/cholesterol mixtures measured by ^2H NMR*
See Materials and Methods for theory and definitions and Table 1 for sample composition

Sample	Temperature	$\Delta^2 H_{obs}$	Phase
	°C	kHz	
IIb	−10	–	no splittings observed
	−1	1.41	lamellar
	28	1.78	lamellar
	43	1.86	lamellar
	58	–	phase transition
IIc	−6	1.45	lamellar
	28	1.52	lamellar
	40	1.53	lamellar
	50	–	phase transition
IIIb	−1	1.56	lamellar
	28	1.82	lamellar
	43	1.88	lamellar
	58	–	phase transition
IIIc	−6	1.51	lamellar
	28	1.60	lamellar
	40	1.79	lamellar
	50	1.91/0.76	lamellar + hexagonal
	70	0.61	hexagonal
Va	−1	1.36	lamellar
	28	1.61	lamellar + cubic
	37	1.43	lamellar + cubic
	43	–	cubic (+ lamellar)[a]
	58	–	cubic (+ lamellar)[a]
Vb	−6	1.30	lamellar
	12	1.67/0.55	lamellar + hexagonal
	28	1.35/0.47	lamellar + hexagonal
	40	0.47	hexagonal
	50	–	hexagonal (not resolved)
VIb	−6	1.43/0.40	lamellar + hexagonal
	−1	1.64/0.38	lamellar + hexagonal
	12	0.40	hexagonal
	28	0.48	hexagonal
	37	0.48	hexagonal
	43	–	hexagonal (not resolved)
	58	–	hexagonal (not resolved)

[a] Contains small amounts of lamellar phase.

Palmitoyloleoyl Glucolipids plus Cholesterol

Incorporation of cholesterol in *A. laidlawii* membranes with palmitoyloleoyl lipids has no significant effect on the $Acyl_2GlcGro/Acyl_2Glc_2Gro$ ratio [11]. The composition in samples IIb (1/1) and IIIb (2/1) (27% cholesterol both) thus can occur *in vivo*. The cholesterol content in samples IIc (1/1) and IIIc (2/1), i.e. 50% (mol/mol), is much higher than that found in *Acholeplasma* species.

All palmitoyloleoyl glucolipids with 27% or 50% cholesterol had lamellar phase structures in the cellular growth temperature range (Table 2). Polarizing light microscopy studies of samples IIc and IIIc (50% cholesterol both) at 25 °C revealed a fine mosaic texture typical for a lamellar phase [23]. In addition, bright, almost square crystals of cholesterol cf. [24] were observed. This shows that the glucolipids cannot dissolve 50% (mol/mol) cholesterol at 25 °C. The bright

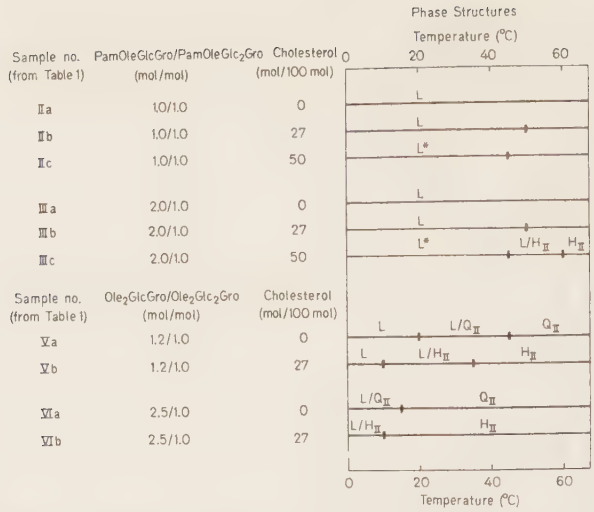

Fig. 2. *Phase equilibria of* Acholeplasma laidlawii *membrane glucolipid/cholesterol mixtures.* A summary of NMR and polarizing light microscopy results. L, lamellar phase; Q_{II}, reserved cubic phase; H_{II}, reversed hexagonal phase. * Samples containing free cholesterol

crystals disappeared gradually when heating the samples, and at 60 °C a one-phase region of fine mosaic texture was obtained. NMR investigations revealed a phase transition around 50–60 °C for samples IIb, IIc and IIIb. In sample IIIc (2/1) at 50 °C small amounts of a hexagonal phase were present in addition to the lamellar phase and at 70 °C a hexagonal phase structure only was detected (Table 2).

Dioleoyl Glucolipids without Cholesterol

For dioleolyl lipds, the $Acyl_2GlcGro/Acyl_2Glc_2Gro$ ratio in sample IV (0/1) does not occur *in vivo*, the ratio in sample Va (1.2/1) is slightly higher, and the ratio in sample VIa (2.5/1) much higher than that observed *in vivo*.

An $Ole_2GlcGro/Ole_2Glc_2Gro$ 1.2/1 mixture (sample Va) could only form a lamellar phase at low temperatures (Table 2). With increasing temperature a mixture of lamellar plus cubic phases, and eventually a cubic phase was observed. With a glucolipid ratio of 2.5/1 (sample VIa) the cubic phase dominated in the physiological temperature range [15].

Dioleoyl Glucolipids plus Cholesterol

Cholesterol supresses the synthesis of $Acyl_2GlcGro$ in membranes with dioleoyl lipids [13]. The glucolipid ratios in sample Vb (1.2/1) and VIb (2.5/1) are thus far higher than those observed together with cholesterol *in vivo*.

A glucolipid ratio of 1.2/1 plus 27% cholesterol (sample Vb) gave a lamellar phase region at very low temperatures only (Table 2). In the growth temperature range (approx. 10–43 °C) a mixture of lamellar and hexagonal phases or a hexagonal phase occurred (Table 2). The cubic phase occurring with this lipid ratio without cholesterol (sample Va) was not observed with 27% cholesterol. Polarizing light microscopy revealed very few cholesterol crystals at 25 °C, which vanished on slight heating. The texture was mosaic plus non-striated, typical for lamellar and hexagonal phases respectively [23], in accordance with our NMR data. At 40 °C a one-phase non-striated (i.e. hexagonal phase) appearance was evident.

A glucolipid sample with a ratio of 2.5/1 and 27% cholesterol (sample VIb) formed a mixture of lamellar and hexagonal phases at low temperatures (Table 2). At physiological temperatures a hexagonal phase was found. The cubic phase occurring with this lipid ratio without cholesterol (sample VIa) was not observed. In the polarizing light microscope very few cholesterol crystals were seen at 25 °C and the texture was non-striated (i.e. hexagonal phase). At 50 °C a pure hexagonal phase was observed.

The splittings of the hexagonal phase in samples Vb and VIb were between three and four times smaller than those for the lamellar phase (Table 2), in accordance with deuteron NMR studies of pure $Acyl_2Glc_2Gro$ (lamellar) and $Acyl_2$-GlcGro (H_{II}) [2], cf. Materials and Methods.

DISCUSSION

The geometry of the lipid molecules has lately been recognized to play a decisive role in determining the phase structures formed by different amphiphiles [1,16]. The packing constraints in a lipid mixture can be modified in two ways: (a) by changing the proportions of different lipids having dissimilar molecular geometries; and (b) by changing the geometry of the lipid molecules themselves in a given mixture. In this work both possibilities have been exploited by varying the amount of cholesterol, $Acyl_2GlcGro$ and $Acyl_2Glc_2Gro$ in the mixtures, as well as the temperature of measurement and the acyl chain unsaturation of the glucolipids.

Fig. 2 shows great differences in phase structures formed by the lipid mixtures with equal amounts of palmitoyl and oleoyl chains and the mixtures containing oleoyl chains only. The former mixtures formed lamellar phases within the growth temperature range of *Acholeplasma laidlawii*, while

cubic and hexagonal phase structures were predominant in the latter mixtures. We conclude that the glucolipid mixtures with nearly 100% *cis*-unsaturated acyl chains are considerably more sensitive to the destabilizing effect of cholesterol, $Acyl_2$-GlcGro, and high temperatures on the lamellar phase. This conclusion is logical and easily explained by the self-assembly theory by Israelachvili et al. [1,14,16]. The above-mentioned parameters together with increased *cis* unsaturation make the lipid molecules more wedge-shaped, and consequently more prone to form a curved aggregate than a planar bilayer structure. The cubic and hexagonal structures found in the present work are most likely of the reversed type (i.e. Q_{II} and H_{II}) because of the small amounts of water present, cf. [2,15].

Interestingly, the cubic structure was formed by pure glucolipid mixtures, while the hexagonal structure was formed by the same mixtures supplemented with cholesterol (Fig. 2). The cubic phase is thus proposed to constitute an 'intermediary' phase between the lamellar and the H_{II} phase. The effect of changing the temperature is most obvious for the lipid mixtures containing oleoyl chains only. The lamellar phase is gradually abandoned for the benefit of a Q_{II} or H_{II} phase. Moreover, this phase transition is moved towards lower temperatures when the amounts of cholesterol and $Acyl_2$-GlcGro are increased. Similar results have been obtained by Cullis and coworkers with phospholipids. A transition from a lamellar to an H_{II} phase structure was evoked by increasing the proportion of soya phosphatidylethanolamine in mixtures with egg-yolk phosphatidylcholine [4], by increasing the temperature or cholesterol concentration in Ole_2Gro-*P*-Etn/Ole_2Gro-*P*-Cho mixtures [25] or by increasing the Ca^{2+}/cardiolipin ratio from 0 to 1.0 [26]. In all cases the transition proceeded via a phase structure characterized by narrow peaks in the NMR spectra.

The results obtained from the lipid mixtures *in vitro* can be compared with the physiological regulation of the $Acyl_2$-GlcGro/$Acyl_2Glc_2$Gro ratio and acyl chain composition exhibited by *A. laidlawii* cells. Incorporation of cholesterol into membranes having lipids with oleoyl chains gives a strong decrease in the synthesis of $Acyl_2$GlcGro and an increase in the synthesis of ionic lipids [11,13]. These metabolic responses counteract the bilayer-destabilizing effect of cholesterol [14]. When the sterol is added to an *A. laidlawii* culture grown with an equimolar mixture of palmitic and oleic acid the synthesis of $Acyl_2$GlcGro is hardly affected. In this case a response is not motivated by lipid-phase structural demands (Fig. 2).

Small alterations in the geometry of the lipid molecules can impose considerable disturbances in lipid packing properties. A difference of only 10% in saturated acyl chain content between synthetic dielaidoylglycerophosphoethanolamine and elaidate-enriched phosphatidylethanolamine from *Escherichia coli* raises the temperature for the lamellar to H_{II} phase transition at least 30°C upwards in the *E. coli* phosphatidylethanolamine as compared to the synthetic phosphatidylethanolamine [27]. However, both lipids show a gel to liquid-crystalline phase transition around 35–37°C.

The lipid mixtures with equal amounts of palmitoyl and oleoyl chains maintain the lamellar phase in the growth temperature range even in presence of 50% (mol/mol) cholesterol (Fig. 2). However, the polarizing light microscopy results indicate that the maximum solubility of cholesterol in these mixtures is close to 27% (mol/mol), which approximately corresponds to the maximum incorporable amount in *A. laidlawii* membranes. Higher cholesterol concentrations give a free cholesterol phase, which is probably fatal for a biological membrane.

The difference in membrane lipid composition between the *Acholeplasma* and *Mycoplasma* species may explain why the latter genus tolerates higher concentrations of cholesterol in its membranes. The solubility of cholesterol in the *A. laidlawii* glucolipids is relatively low. Compared to the *Acholeplasma* species the *Mycoplasma* species have significant differences in lipid content.

a) Smaller amounts of lipids forming an H_{II} phase [28]; such lipids more easily form non-lamellar phases together with cholesterol (Fig. 2).

b) Larger amounts of anionic lipids are present [28]; the net charge of, for example, phosphatidylserine influences the phase structure formed by the lipid [29]. Above pH = 4.0 phosphatidylserine is negatively charged and adopts a lamellar phase; however, if the pH is lowered to 3.0 the carboxyl group is protonated. The lipid becomes uncharged and forms an H_{II} phase structure [29]. Thus, at physiological pH values anionic lipids have a greater capability than non-ionic lipids to stabilize the bilayer configuration.

c) Small amounts of lysolipids [28], which form normal hexagonal (H_I) phases [1], are present. Incorporation of lysolecithin into liposomes of egg-yolk phosphatidylcholine decreases the ability of the liposomes to trap glucose [30]. Cholesterol incorporation counteracts this effect and an equimolar mixture of cholesterol and lysolecithin can by itself form a stable bilayer [30]. All the above-mentioned factors neutralize the effect of the wedge shape of cholesterol and allow higher concentrations of this lipid in the membranes of *Mycoplasma* species compared to *Acholeplasma* species. Cholesterol must be important to the former, since adaptation of *Mycoplasma mycoides* to growth with little cholesterol makes the cells greatly increase the amount of diacylglycerides in their membranes [31]. Cholesterol and diacylglycerides have similar wedge shapes. However, because of the phylogenetic distance between the two genera [32] there may be additional causes for the high cholesterol tolerance of the genus *Mycoplasma*. Finally, a parallel can be drawn between the occurrence of phosphatidylethanolamine/cholesterol and monosugar diacylglycerides/cholesterol in biological membranes. Monosugar diacylglycerides are present in larger amounts in *Acholeplasma* species than in *Mycoplasma* species [28], while the opposite is true for cholesterol.

It should be noted that in the discussions of the reported experiments the effect of cholesterol has not been explained by the concept of 'fluidity'. Incorporation of cholesterol into model bilayer [33] and *A. laidlawii* [34–36] membranes increase the order parameter of the hydrocarbon chains significantly but leaves the lipid lateral diffusion almost unaffected [37]. We thus conclude that the effect of cholesterol on the packing properties of the bilayer is more important than its influence on lipid bilayer dynamics.

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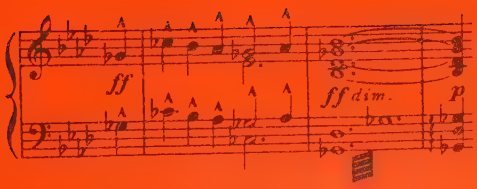
III



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Reversed Cubic Phase with Membrane Glucolipids from *Acholeplasma laidlawii*. ^{1}H, ^{2}H, and Diffusion Nuclear Magnetic Resonance Measurements†

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ABSTRACT: Monoglucosyl diglyceride and diglucosyl diglyceride are the dominant lipids of the *Acholeplasma laidlawii* membrane. Diglucosyl diglyceride forms a lamellar liquid crystalline phase with water while monoglucosyl diglyceride forms a reversed hexagonal phase. Depending on the amounts of unsaturated acyl chains of the lipids, a *mixture* of monoglucosyl diglyceride and diglucosyl diglyceride forms lamellar or reversed cubic phases at physiological temperatures. A high degree of cis unsaturation favors formation of the cubic phase with increasing monoglucosyl diglyceride content. The structure of the cubic phase is composed of aggregates, where the lipids can diffuse over macroscopical distances. A structure containing close-packed spherical *micelles* is therefore ruled out, and the NMR diffusion data are compatible with other previously proposed cubic bicontinuous structures [Luzzati, V., & Spegt, P. A. (1967) *Nature (London) 215*, 701; Scriven, L. E. (1976) *Nature (London) 263*, 123; Lindblom, G., Larsson, K., Johansson, L. B.-Å., Fontell, K., & Forsén, S. (1979) *J. Am. Chem. Soc. 101*, 5465]. Monoglucosyl diglyceride/diglucosyl diglyceride ratios forming cubic phases have not been observed in vivo. It is concluded that formation of the cubic phase is strongly dependent on the molecular shape of the lipids. The results are significant for the physiological regulation of the lipid composition in *A. laidlawii* membranes as well as for the function and organization of biological membranes in general.

All biological membranes contain a number of different lipids (Ansell et al., 1973), most of which form lamellar liquid crystalline phases together with water (Luzzati & Tardieu, 1974). However, exceptions to formation of the bilayer structure have been found for some lipids. Hence it has been shown that phosphatidylethanolamine (PE)[1] from various organisms (Shipley, 1973) and monoglucosyl diglyceride (MGDG) from *Acholeplasma laidlawii* (Wieslander et al., 1978) form reversed hexagonal (H_{II}) mesophases. The only lipid structure compatible with a nonleaky and functioning membrane is the lamellar phase, and lipid bilayer stability thus is of crucial importance for the living cell. It cannot be excluded, however, that other mesophase structures may form within the membrane during short time periods or in the proximity of integral membrane proteins. Local regions forming such structures have been proposed to be advantageous to cell functions like membrane fusion (Lucy, 1975), exo- and endocytosis, and transbilayer movement of lipids (Cullis & De Kruijff, 1979). The very rapid translocation of PE from the inner to the outer leaflet in *Bacillus megaterium* membranes has been shown to be independent of the synthesis of lipid and protein and of sources of metabolic energy, probably excluding a "flip-flop" mechanism (Langley & Kennedy, 1979). Instead, lateral diffusion along transient lipid "hairpin" structures in the vicinity of hydrophilic transmembrane channels is suggested. Since the hairpin bend has a small radius of curvature, it must be stabilized by lipids forming highly curved aggregates (Langley & Kennedy, 1979). However, large amounts of such lipids destabilize the membrane, eventually leading to a membrane disrupture. Therefore, the balance between lipids forming lamellar and nonlamellar phases can only vary within certain limits in order to

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[1] Abbreviations used: MGDG, monoglucosyl diglyceride; DGDG, diglucosyl diglyceride; NMR, nuclear magnetic resonance; H_{II}, reversed hexagonal; PE, phosphatidylethanolamine.

keep the bilayer stable (Wieslander et al., 1980).

In bacteria the membrane often has to cope with various factors affecting the bilayer stability. An increase in temperature might lead to the formation of an isotropic phase and, on the other hand, a decrease in temperature may induce a lipid crystalline (gel) phase. At constant temperatures, an increase in the amount of "nonlamellar lipids" above a critical concentration destabilizes the membrane. Such events must be counteracted by the cell, and mechanisms must exist regulating lipid properties in the membrane so that optimal stability always is achieved. These regulations occur in the membrane of *A. laidlawii*, and recently we showed that lipid composition in this membrane to a large extent is determined by the shape or the molecular geometry of the various lipids (Wieslander et al., 1980).

In this work we have studied the liquid crystalline phase behavior of different mixtures of the two dominant polar lipids in *A. laidlawii*, MGDG, and diglucosyl diglyceride (DGDG). It is found that the amount of unsaturated acyl chains in the lipids is of utmost importance for the phase structure formed. Here we report the first observed cubic lyotropic liquid crystalline phase for a mixture of membrane lipids at *low temperatures*. Previously, cubic phases for lecithin (Small, 1967; Luzzati, 1968) and for chloroplast galactolipids at high temperatures (Rivas & Luzzati, 1969) have been reported. The structure of the amphiphilic aggregates building up cubic phases have been determined for some systems by using X-ray (Luzzati & Spegt, 1967; Lindblom et al., 1979) and NMR techniques (Lindblom et al., 1979). Here is shown that the structure of the cubic phase observed for the mixture of *A. laidlawii* glucolipids consists of continuous hydrocarbon regions.

Materials and Methods

Membrane Lipids. Acholeplasma laidlawii A, strain EF 22, was grown statically in a lipid-depleted bovine serum albumin–tryptose medium (Wieslander et al., 1978). To obtain lipids containing only cis-unsaturated acyl chains, the growth medium was supplemented with 150 μM oleic acid (Wieslander & Rilfors, 1977; Christiansson & Wieslander, 1980) and the cells were harvested after growth for 36 h at 30 °C. This temperature has the effect of increasing the relatively low amounts of MGDG normally obtained with oleic acid supplementation (Christiansson & Wieslander, 1978). Lipids containing approximately equal amounts of an unsaturated and a saturated acyl chain were obtained by supplementing the medium with 75 μM oleic acid and 75 μM palmitic acid and growing the cells for 24 h at 37 °C (Wieslander & Rilfors, 1977; Christiansson & Wieslander, 1980). Preparation of membranes, purification, and analysis of single lipid species were done as described previously (Wieslander et al., 1978). Typically, a 10-L batch yielded 525 mg of total lipids.

Sample Preparation. Different mixtures of MGDG and DGDG, approximately 90 mg of total lipids, were prepared by careful weight analysis. The solvents were removed by N_2 and by drying the samples overnight at a pressure less than 0.1 mmHg. Dissociable protons in the lipid polar head groups were exchanged for deuterons by vigorously mixing the dried samples with excess ^2H_2O in an atmosphere of N_2 and thereafter removing the heavy water by drying as described above. This procedure was carried out three times. Finally 11 or 13% (w/w) of ^2H_2O was added, followed by a flush of N_2, and mixed with the lipids by extended centrifugation. The heavy water concentrations used are slightly lower than the maximum hydration capacities of these lipids (Wieslander et al., 1978). Water concentrations above this limit were avoided since two-phase samples may complicate the interpretation of the NMR spectrum. Moreover, MGDG and DGDG form H_{II} and lamellar phase structures, respectively, in excess water (Wieslander et al., 1978), and cubic liquid crystalline phases have been shown to be in equilibrium with very dilute aqueous solution phases (Fontell, 1978). Preparation and the macroscopic alignment (De Vries & Berendsen, 1969; Lindblom, 1972) of the lamellar samples between cleaned glass plates were performed on a heated metal plate in a plastic box containing N_2 and ^2H_2O vapor at 35 °C.

^1H and ^2H NMR. ^2H NMR spectra were recorded at 15.351 MHz on a Varian XL 100 NMR spectrometer operating in the Fourier transform mode. For an oriented lamellar sample, the ^2H NMR spectrum consists of a doublet, a quadrupole splitting given by (Wennerström et al., 1974)

$$\Delta(\theta) = |\nu_Q S(3 \cos^2 \theta - 1)|$$

where θ is the angle between the external magnetic field, B_0, and the axis of alignment (the director), ν_Q is the effective quadrupole coupling constant, and S is the order parameter, describing the average orientation of the water molecules in the mesophase. Thus, if the lamellar phase is aligned along the glass plates, the splitting is largest when the plates are perpendicular to the magnetic field ($\theta = 0°$) and Δ has half this value when the plates are parallel with the magnetic field ($\theta = 90°$). This was also observed as illustrated in Figure 1. The temperature dependence of the splittings was 3% between 30 and 45 °C. For a H_{II} phase aligned between glass plates, the deuteron NMR spectrum will have quite a different pattern (Mely et al., 1975; Söderman et al., 1980). For an isotropic phase, like a cubic phase, no splitting is usually observed (Figure 1d). As a complement, proton NMR spectra were recorded at 100 MHz (Figure 1e).

NMR Diffusion. The lipid diffusion coefficients were measured at 61 MHz on a Bruker 322s spin-echo NMR spectrometer using a pulsed magnetic field gradient technique (Stejskal & Tanner, 1965). The spectrometer was equipped with a home-built pulsed magnetic field gradient unit with digital settings for all experimental parameters used. A schematic illustration of the experiment is presented in Figure 2. The diffusion coefficient is obtained from the attenuation of E_g/E_0 of a spin echo by varying the strength of the magnetic field gradients (δ or g) or the distance, Δ, between them according to

$$\ln \frac{E_g}{E_0} = -(\gamma g \delta)^2 D(\Delta - \tfrac{\delta}{3})$$

where δ is the width and g the height of the magnetic gradients, γ is the gyromagnetic ratio, and D is the diffusion coefficient. Typical settings for a lamellar sample were Δ = 12 ms, δ = 2 ms, and g varied between 0 and 3.5 T m^{-1}. For a cubic mesophase sample, δ and Δ were chosen larger, 5 and 30 ms, respectively. The temperature for all samples was 45 °C. All measurements were performed relative to glycerol reference with a known diffusion coefficient (Tomlinson, 1973).

For determination of the lateral diffusion coefficient of a bilayer, the lamellar sample has to be macroscopically aligned and put at the so-called "magic angle" in the magnetic field ($\theta = 54.7°$), as illustrated in Figure 2. This technique for measurement of lipid lateral diffusion has been shown to be very useful and has been practiced in several previous studies by us (Lindblom & Wennerström, 1977; Wennerström & Lindblom, 1977; Lindblom et al., 1977, 1979; Arvidson et al., 1978; Söderman et al., 1980) and others (Tiddy, 1977; Kuo & Wade, 1979). For details of the method, see Lindblom & Wennerström (1977).

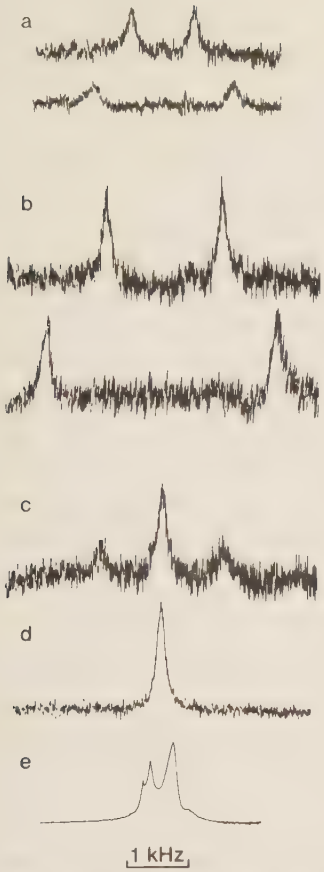

FIGURE 1: (a–d) Deuteron NMR spectra; (e) proton NMR spectrum. (a) Macroscopically aligned lamellar sample of DGDG (16:0/18:1c). Upper spectrum: the magnetic field, B_0, is parallel with the glass plates. Lower spectrum: B_0 is perpendicular to the glass plates. Note the difference in the quadrupole splitting equal to 2. (b) Macroscopically aligned lamellar sample of DGDG (18:1c/18:1c). Upper and lower spectra as in (a). (c) Macroscopically aligned two-phase sample containing lamellar (splitting) and cubic (central peak) mesophases. Molar ratio MGDG/DGDG (18:1c/18:1c) is equal to 1.2. (d) Cubic liquid crystalline phase with molar ratio MGDG/DGDG (18:1c/18:1c) equal to 2.5. (e) Proton NMR spectrum of the sample in d. All spectra were recorded at 30 °C. Reproducibility of the spectral shapes was complete when investigated at intervals of a month. The standard deviation for the quadrupole splittings is better than ±5%.

Results

The composition of the samples studied are summarized in Table I. Two series of samples were employed having (a) variations in the molar ratio between MGDG and DGDG from 0 to 2 (in this series about half of the acyl chain content was cis unsaturated) and (b) similar changes as in (a) of the ratio MGDG/DGDG but all (>95%) of the lipid acyl chains were cis unsaturated. A glucolipid composition like that in samples I and IV has not been observed in vivo. The composition of samples II and III occurs in vivo, but only with this acyl chain composition (Wieslander & Rilfors, 1977; Christiansson & Wieslander, 1978, 1980; Wieslander et al., 1979). For lipids containing oleic acyl chains only, the MGDG/DGDG ratio in sample V was slightly higher, and in sample VI it was much higher than what has been observed in vivo.

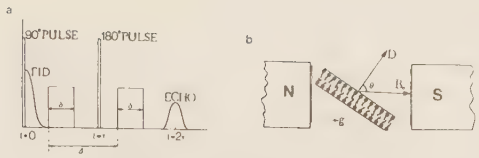

FIGURE 2: (a) Schematic picture of the NMR diffusion method with 90–τ–180 rf pulses and pulsed magnetic field gradients. δ is the width and Δ the spacing between the field gradient pulses. FID, free induction decay. (b) Illustration of the orientation of the macroscopically aligned lamellar sample relative to the external magnetic field, B_0, and the magnetic field gradient, g.

Table I: Composition of Mixtures Made from *A. laidlawii* Membrane Glucolipids

sample	MGDG/DGDG[a]	acyl chain composition		
			16:0[b]	18:1c[b]
I	0:1.0	MGDG	54	46
II	1.0:1.0	DGDG	45	55
III	2.0:1.0			
IV	0:1.0	MGDG and DGDG		
V	1.2:1.0	18:1c[b] ≥ 95		
VI	2.5:1.0			

[a] MGDG and DGDG: mono- and diglucosyl diglyceride; molar relationships in samples. Total amounts of lipids approximately 90 mg. [b] 16:0, palmitic acid; 18:1 c, oleic acid; amounts in mol %.

As can be inferred from Figure 1 the magnitude of the deuteron quadrupole splitting was larger for the samples containing ~100% unsaturated acyl chains than for the corresponding samples with ~50% of the acyl chains being unsaturated. For samples I, II, III, and IV it was found that the deuteron NMR spectra obtained at $\theta = 0°$ and 90° are compatible with lamellar phases (cf. Methods) as shown for samples I and IV in Figure 1. Sample VI showed no quadrupole splitting, but only a narrow single NMR peak (Figure 1d). In sample V, however, a splitting of very low intensity was observed, together with the central high intensive peak (Figure 1c). The splitting was twice as large when the sample was rotated 90° in the magnetic field. The proton NMR spectrum of the sample VI exhibits quite narrow peaks (Figure 1e), indicating that it consists of an isotropic phase. A lamellar phase would give a broad and unressolved proton NMR spectrum. Such spectra were obtained from samples I, II, III, and IV. Sample VI was found to be optically isotropic when studied between crossed polarizers, but sample V showed both anisotropic and isotropic regions, i.e., V is a two-phase sample. The macroscopic viscosity was considerably higher for samples V and VI compared to the other samples. A common feature for optical isotropic liquid crystalline phases is their very stiff consistency (Small, 1967; Fontell, 1978). The hexagonal phases are characterized by a somewhat lower apparent viscosity, and the lamellar phase is much less viscous (Fontell, 1978). All these data taken together lead to the conclusion that samples I, II, III, and IV consist of lamellar liquid crystalline phases, while sample VI is an isotropic cubic liquid crystal and sample V consists of two phases, lamellar and cubic.

In order to gain further information about the structure of the amphiphilic aggregates in the isotropic phase, the diffusion coefficient for some of the samples was determined. The data obtained are given in Table II. Lipid diffusion in lamellar and cubic phases differs by about one order of magnitude (compare the experimentally determined coefficients).

Table II: Experimental and Calculated Values for Lipid Diffusion Coefficients[a]

sample[b]	$D \times 10^{11}$ (m^2/s)	$D_L \times 10^{11}$ (m^2/s)	$D_{\parallel}^{cub}$ (m^2/s)
III	0.8	1.2	
IV	2.6	3.9	
V[c]	0.1		0.3
VI	0.1		0.3

[a] D_L represents the lateral diffusion coefficient in a lipid bilayer, obtained by correcting the experimentally determined diffusion coefficient, D, for the "magic angle". $D_{\parallel}^{cub}$ is the estimated diffusion coefficient for translational motion along an assumed rodlike aggregate unit in a cubic phase. Temperature 45 °C. The standard deviation for D is ±30%. [b] See Table I for sample composition. [c] This sample formed a pure cubic phase at 45 °C.

Discussion

In previous work (Lindblom & Wennerström, 1977; Arvidson et al., 1978; Lindblom et al., 1979) it has been shown that from a comparison between diffusion coefficients for lamellar and cubic phases, determined by the NMR diffusion technique, information about the aggregate structure in the cubic phase can be obtained. Conclusions made are based on the fact that the value of the apparent diffusion coefficient depends strongly on the overall structural geometry. For a discussion of the method in detail the reader is referred to the works cited above. Here only a brief account will be given. For a cubic phase composed of closepacked spherical aggregates, the lipid translational motion is confined to the globular micelle (exchange between aggregates can be neglected), and therefore the measured diffusion coefficient will be very low. It has been found that this coefficient for such a cubic phase is more than two orders of magnitude lower than for the corresponding lamellar phase. For a cubic phase based on rod network systems (Luzzati & Spegt, 1967), lipid diffusion can occur over macroscopial distances, and the measured lipid diffusion coefficient for such a phase is approximately of the same order of magnitude as the value obtained for the lamellar phase. A quantitative comparison between measured and calculated diffusion coefficients in the different phases is often possible to make.

From the experimental findings obtained for the lamellar samples III and IV, it can be inferred (Table II) that the lateral diffusion coefficients are of the same order of magnitude, being about a factor three lower for sample III. This is in line with previous results where it was found that the lipid lateral diffusion depends on the polar head group area at the water–lipid interface, i.e., on the packing properties of the bilayer (Lindblom & Wennerström, 1977). Thus the more saturated lipids in sample III can pack together much tighter than the cis-unsaturated lipids in sample IV. An increase of the polar head group area with increasing amounts of cis-unsaturated acyl chains of the lipids is supported by the water deuteron NMR data in Figure 1. The larger splitting obtained for the more unsaturated lipids is most likely due to an increase in the water binding to the head-group region of the bilayers. Such an increase will occur because of the increase in space between the lipid molecules.

Recently we studied the lateral diffusion of some different lecithins (Lindblom et al., 1980), making it possible here to consider also the effect of a change in the polar head group on the translational lipid motion in a bilayer. The lateral diffusion coefficients of palmitoyloleoyllecithin and dioleoyllecithin were found to be nearly one order of magnitude lower than for the glucolipid lamellar samples with corresponding acyl chain composition. The most likely explanation of this is, in our opinion, that the packing and electrostatic properties are very different in the phospho- and glucolipid systems in question. It should be noted that the gel to liquid crystalline phase transition temperature is approximately 20 °C higher for the *A. laidlawii* glucolipids (Wieslander et al., 1978) than for the corresponding lecithins.

Table II shows that for samples V and VI, containing cubic phases, the experimentally determined diffusion coefficient is not more than one order of magnitude lower than for the lamellar samples III and IV. It is obvious from a comparison between the diffusion coefficients obtained for the lamellar and the cubic mesophases that the latter phase cannot be composed of spherical aggregates (cf. above), but must contain *continuous hydrocarbon regions*. Our results are compatible with the cubic phase structure proposed by Luzzati & Spegt (1967). Since the water content is only 11% (w/w), the cubic phase most likely is of the reversed type. It can be pictured as two distinct three-dimensional networks of water rods in a lipid matrix, which are unconnected but mutually interwoven [see Figure 7 in Fontell (1978)]. However, other types of cubic phases containing continuous hydrocarbon regions cannot be totally ruled out. A cubic phase formed by a network of hexagon-shaped lamellar bilayer units has been found in a mixture of monoolein and water (Lindblom et al., 1979), and Scriven (1976) has described some additional continuous cubic lattices.

The phase structures formed by the glucolipids can be compared to those formed by the structurally close related galactolipids. With galactolipids isolated from wheat endosperm flour, monogalactosyl diglyceride/digalactosyl diglyceride ratios ranging from 0.6 to 2.5 resulted in a two-phase region at low water contents, consisting of a lamellar and a H_{II} phase (Larsson & Puang-Ngern, 1979). Higher and lower ratios gave pure H_{II} and lamellar phases, respectively. Rivas & Luzzati (1969) investigated a galactolipid mixture isolated from maize chloroplasts. This mixture formed a H_{II} phase from −20 to 100 °C when the water content was below 10% (w/w) and a cubic phase, consisting of two interwoven three-dimensional rod networks, with water concentrations between 10 and 20% (w/w) and at temperatures between 60 and 100 °C. However, this lipid preparation also contained some sulfolipids, which form a lamellar phase with water (Shipley et al., 1973). Moreover, the degree of unsaturation was different in the two lipid preparations, which has been shown in this work to influence the phase structures formed by mixtures of the glucolipids.

The cubic liquid crystalline phase can form with a mixture of MGDG and DGDG when (a) MGDG is present in excess and (b) the content of cis-unsaturated acyl chains is approximately 100%. The area occupied by the polar head group of MGDG is smaller than that occupied by DGDG. Morever, a cis-unsaturated acyl chain cannot extend to the same length as a saturated one (Träuble & Haynes, 1971). The above-mentioned factors thus affect the *mean* polar head group area at the hydrocarbon–water interface and the hydrocarbon chain length in the lipid aggregates. According to the theory of self-assembly of amphiphiles developed by Israelachvili et al. (1976; Israelachvili 1977), these quantities determine the shape of the lipid molecules and consequently the structure of the amphiphilic aggregates that are formed. MGDG has a more or less pronounced wedge-like molecular shape with different acyl chain contents, and forms a H_{II} phase structure with water. DGDG, having a rodlike molecular shape, forms a lamellar phase (Wieslander et al., 1978). In 1:1 and 2:1

mixtures of MGDG and DGDG with about 50% of the acyl chains being cis unsaturated, lamellar phases were formed. DGDG thus has the capacity to stabilize the lamellar phase with this acyl chain composition. When the content of cis-unsaturated acyl chains was increased to nearly 100%, a lamellar *and* a cubic mesophase was formed with the MGDG/DGDG ratio being 1.2:1, and a *pure* cubic phase was formed with the 2.5:1 MGDG/DGDG mixture. An increase in the fraction of cis-unsaturated acyl chains will increase the hydrophobic bulkiness of both the glucolipid molecules, leading to more wedgelike molecular shapes and enhanced tendencies to form nonlamellar phases (Wieslander et al., 1980). The bilayer stabilizing property of DGDG is diminished, and when MGDG is present in great excess the wedge shape properties are accentuated to such an extent that the lamellar phase is totally abolished.

The results obtained are highly relevant to the regulation of membrane lipid composition observed in *Acholeplasma laidlawii* grown under different conditions. In particular, the ratio between MGDG and DGDG is altered depending on configuration of incorporated fatty acids, growth temperature, and membrane cholesterol content (Wieslander & Rilfors, 1977; Christiansson & Wieslander, 1978, 1980). These factors affect the molecular geometry of the membrane lipids (Israelachvili et al., 1976, Israelachvili, 1977; Wieslander et al., 1980). Incorporation of different ratios of saturated/unsaturated fatty acids in *A. laidlawii* membranes causes a successive change in the hydrophobic geometry of the lipids. When the amount of cis-unsaturated acyl chains in the membrane increases, the organism maintains the membrane stability by substantially decreasing the amount of MGDG, the polar lipid exerting the greatest bilayer destabilizing effect in *A. laidlawii* membranes. The MGDG/DGDG ratio has a maximum value of 0.8 in membranes, being totally enriched in oleic acid (Christiansson & Wieslander, 1978), a value which is somewhat *lower* than that in sample V, giving a mixture of a cubic and a lamellar phase. A larger hydrophobic bulkiness of the lipid molecules is likewise generated by raising the temperature. The effect is counteracted in *A. laidlawii* by supressing the wedge shape properties of these molecules in two ways: (1) reduced incorporation of unsaturated fatty acids and (2) decreased synthesis of lipids with a small polar head group like MGDG. Finally, the MGDG content in the *Acholeplasma* membrane containing oleic acyl chains is strikingly reduced by incorporation of cholesterol. The latter molecule destabilizes the bilayer structure in the presence of lipids, forming a H_{II} phase, because of its marked wedge shape (Carnie et al., 1979). The effects of temperature and cholesterol on the phase structure of MGDG/DGDG mixtures have also been investigated, and the results further support the conclusion that the MGDG/DGDG ratio has a bilayer stability regulating function (A. Khan, Å. Wieslander, L. Rilfors, and G. Lindblom, unpublished results).

In conclusion, samples with MGDG/DGDG ratios and an acyl chain composition occurring in vivo (II, III) clearly revealed stable lamellar phases. The sample with a composition just outside the range found in vivo (V) gave a two-phase system consisting of a lamellar and a cubic phase. When the composition was far removed from that observed in vivo (VI), a pure cubic phase was obtained. Since the other polar lipid species isolated from the membrane of *A. laidlawii* strain A form lamellar phases (Wieslander et al., 1978), it can be assumed that the cells, at the prevailing growth conditions, actively avoid lipid compositions resulting in nonlamellar phases of the bulk membrane lipids. It is conceivable, however, that such phases can form temporarily and/or within local regions of biological membranes, since several observations indicate that the lipid species are not uniformly distributed in this organelle: (1) the activities of a limited number of membrane associated enzymes are dependent on a specific lipid surrounding (Sandermann, 1978); (2) there are strong indications of an asymmetrical distribution of lipid species between the bilayer halves in procaryotic as well as eucaryotic cells (Op den Kamp, 1979; Gross & Rottem, 1979); and (3) lipids with specific molecular geometries may be required in order to minimize packing defects around the irregular surfaces of integral membrane proteins (Israelachvili, 1977; Sandermann, 1978). Stepwise selective solubilization of *A. laidlawii* membranes with Tween 20 has shown that a specific protein is enriched together with MGDG (Å. Wieslander and K.-E. Johansson, unpublished results). Indications of the existence of nonlamellar phases within plasma membranes of both eucaryotic and procaryotic cells derives from some ^{31}P NMR investigations (Stier et al., 1978; Cullis & De Kruijff, 1979; Burnell et al., 1980). Most biological membranes contain at least one lipid species having the capacity to form a hexagonal phase. These observations, together with the fact that the synthesis of MGDG never ceases in *A. laidlawii*, favor the proposal that certain amounts of a lipid forming a nonlamellar phase must be beneficial for membrane bilayer properties and some membrane–mediated cell processes. Exo- and endocytosis, membrane fusion, and transbilayer movement of lipids are easier to reconcile with a transient of regional formation of nonlamellar phases than with an invariable preservation of the bilayer structure. Recently it has also been suggested that the transport of nucleic acid into the recipient cell at the early stage of genetic transformation, conjugation, transfection, and viral infection involves a rearrangement of the phospholipid bilayer and the formation of a transmembrane channel (Grinius, 1980).

In this work it has been demonstrated that knowledge about the phase structures formed by membrane lipids in simple lipid–water systems is important in order to understand the molecular organization of biological membranes. As shown for *A. laidlawii*, the biological significance of these studies is particularly evident when physical and physiological investigations are connected to each other.

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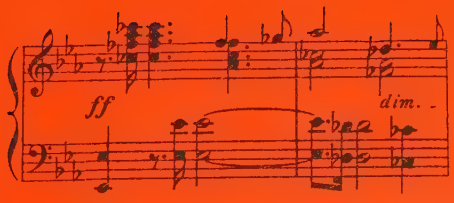
IV



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Lipid and Protein Composition of Membranes of *Bacillus megaterium* Variants in the Temperature Range 5 to 70°C

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Membranes were prepared from four temperature range variants of *Bacillus megaterium*: one obligate thermophile, one facultative thermophile, one mesophile, and one facultative psychrophile, covering the temperature interval between 5 and 70°C. The following changes in membrane composition were apparent with increasing growth temperatures: (i) the relative amount of iso fatty acids increased and that of anteiso acids decreased, the ratio of iso acids to anteiso acids being 0.34 at 5°C and 3.95 at 70°C, and the pair iso/anteiso acids thus seemed to parallel the pair saturated/unsaturated acids in their ability to regulate membrane fluidity; (ii) the relative amount of long-chain acids (C_{16} to C_{18}) increased fivefold over that of short-chain acids (C_{14} and C_{15}) between 5 and 70°C; (iii) the relative amount of phosphatidylethanolamine increased, and this phospholipid accordingly dominated in the thermophilic strains, whereas diphosphatidylglycerol was predominant in the two other strains; and (iv) the ratio of micromoles of phospholipid to milligrams of membrane protein increased threefold between 5 and 70°C. Moreover, a quantitative variation in membrane proteins was evident between the different strains. Briefly, membrane phospholipids with higher melting points and packing densities appeared to be synthesized at elevated growth temperatures.

Most microorganisms alter their membrane composition in response to changes in the environmental temperature. The physical properties of the membrane lipid bilayer are believed to be important for this temperature adaptation. *Escherichia coli* thus maintains a nearly constant fluidity of its membrane lipids over its entire temperature range of growth, a process called "homeoviscous adaptation" (45). The mechanism, in this case, involves the production of membrane lipids containing a higher proportion of saturated fatty acids relative to unsaturated ones at higher temperatures.

Generally, the highest growth temperature for a microorganism is considered to be determined by the thermal destruction of the most heat-labile essential cell component. Membrane integrity has been put forward in one of the theories attempting to explain the phenomenon of thermophily (44). Esser and Souza (13) concluded that for a temperature-sensitive mutant of the obligate thermophile *Bacillus stearothermophilus*, the maximum and minimum growth temperatures were determined by the onset and cessation of the membrane lipid phase separation. However, McElhaney and Souza (30), in a reexamination of this correlation, stated that the upper boundary of the phase transition interval in itself did not directly determine the maximum growth temperature of this thermophile; nor did the lower boundary of the phase transition interval, which occurred well below the growth temperature range, determine the minimum growth temperature of *B. stearothermophilus*, although the minimum growth temperatures of *Acholeplasma laidlawii* and *E. coli* (29, 39, 50) can be determined by this lower boundary. Thus, the thermostability of microorganisms probably involves many factors, and the specific composition of the membrane is but one requirement. McElhaney (29) states that changes in the physical state of the membrane lipids alone will not convert mesophiles into psychrophiles or thermophiles.

The fatty acid compositions of lipids from *Bacillus* species grown at different temperatures have been investigated (4, 8, 22, 44, 47, 52, 56). The species most commonly used was *B. stearothermophilus*. The temperature intervals studied were rather narrow, about 20°C, and the results are conflicting with respect to some fatty acids. Thus, for iso-C_{15}, anteiso-C_{15}, iso-C_{16}, iso-C_{17}, and anteiso-C_{17} fatty acids, increasing as well as decreasing relative amounts with increasing growth temperature were reported. Shen et al. (44) compared six different *Bacillus* species,

and the observed fatty acid composition might have been affected by inherent genetic differences between these species as well as by temperature.

The present investigation used temperature range variants of *B. megaterium* (48). Such variants have extended the capability of temperature adaptation outside the normal limits of their parent bacteria. The temperature-dependent changes in membrane composition could be determined with greater correctness in a genetically homogenous system of strains spanning a growth temperature range of 65°C. Changes in the relative amounts of phospholipid classes, in the average chain length of the fatty acids, in the relative proportions of iso and anteiso acids, and in membrane protein composition were observed in these *B. megaterium* strains when growth temperature was altered.

MATERIALS AND METHODS

Organisms. Four *B. megaterium* strains, designated Fp 1, M 1, Ft R32, and Ot 32, were used throughout this work. Strain Fp 1 is facultatively psychrophilic, strain Ft R32 is facultatively thermophilic, and strain Ot 32 is obligately thermophilic. These strains originate from the same mesophilic wild-type strain, M 1, of *B. megaterium*. Their isolation and characteristics have been described (48). Characteristics of relevance to the present investigation are given in Table 1.

Culture conditions. Stock cultures of the strains were kept on slants of tryptone-starch agar (tryptone [Difco Laboratories, Detroit, Mich.], 10 g; soluble starch [E. Merck AG, Darmstadt, Germany], 2 g; agar, 30 g; distilled water, 1,000 ml) at 4°C. Temperature range stability (Table 1) was tested at different temperatures on this medium before the start of an experiment.

Cultures for inoculum were grown in tryptone-starch broth and transferred at least twice at the temperature intended for the main experiment. Erlenmeyer flasks (1 liter) containing 250 ml of broth were inoculated with 2% (vol/vol) of the preculture and incubated in thermostatically controlled water baths (±0.1°C) with reciprocal shaking. The flasks were equipped with side tubes for turbidimetric measurements, which were made periodically. In all experiments, the cultures were harvested in the mid-exponential growth phase at the same turbidity reading.

The facultative psychrophile was grown at 5, 10, 25, and 35°C; the mesophile was grown at 10, 15, 25, 35, and 44°C; the facultative thermophile was grown at 17, 25, 35, 45, and 55°C; and the obligate thermophile was grown at 40, 50, 55, 60, 65, and 70°C.

Membrane preparation. Cell membranes were prepared by the relatively rapid procedure of Konings et al. (25), which circumvents the usual protoplast formation step. According to this method, the cells are treated with lysozyme in a hypotonic medium. The cell wall is thereby partially hydrolyzed, which results in an immediate lysis of the cells and formation of membrane vesicles. Added deoxyribonuclease and ribonuclease hydrolyze the liberated DNA and RNA. Cell wall remnants are further hydrolyzed by continued incubation in the presence of lysozyme. The membranes were collected by centrifugation for 30 min at 34,000 × *g* (at 4°C). No intact cells were present in the membrane preparations when examined by phase-contrast microscopy. The protein content of membranes was estimated by the method of Lowry et al. (27) modified according to Hartree (16). Bovine serum albumin (Cohn V) was used as a standard.

Polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was performed as described by Neville (35) with the modifications of Jergil and Ohlsson (18). The gels were cast in 9.5- by 7.5- by 0.3-cm slab gel forms. Membrane fractions containing 120 μg of protein were brought to a volume of 25 μl by addition of deionized water. An equal volume was then added of double-strength upper reservoir buffer containing sodium dodecyl sulfate, β-mercaptoethanol, ethylenediaminetetraacetate, sucrose, and phenol red. The samples were placed in a boiling-water bath for 3 min. After cooling to room temperature, the entire sample was placed in a sample well of the slab gel. Electrophoresis was performed at a constant current of 20 mA per slab gel and was terminated just before the phenol red tracking dye migrated out of the gel. The gels were stained for 12 h with 0.05% (wt/vol) Coomassie brilliant blue R 250 in methanol-acetic acid-water (50:7:43, vol/vol) and destained for 48 h in methanol-acetic acid-water (25:7:68, vol/vol). Reference proteins used for molecular weight determinations were: bovine serum albumin (68,000), ovalbumin (43,000), deoxyribonuclease I (31,400), trypsin (23,300), and lysozyme (14,300).

An Eppendorf photometer equipped with a gel and photo scanner was used for scanning the negatives of

TABLE 1. *Genealogy, cardinal temperatures, and important characteristics of the B. megaterium variants*[a]

Strain	Derivation	Cardinal temp (°C)			Characteristic	
		Maximum	Optimum	Minimum	Megacin producer	Phage sensitive
Ot 32	Spontaneous from M 1 at 60°C	73	64	38	−	+
Ft R32	Acridine revertant from Ot 32	58	50	16	+	+
M 1	Mesophilic wild type of *B. megaterium*	45	36	8	−	+
Fp 1	Spontaneous from M 1 at 5°C	42	35	0	−	+

[a] Data taken from reference 48.

gel photos. The qualitative transmission was recorded, and areas under peaks were estimated from the weights of the cut out paper recordings.

Lipid extraction. Lipids were extracted from membrane suspensions according to a modification of the method of Bligh and Dyer (3). Each 1 ml of membrane suspension, containing 1 to 3 mg of protein, was mixed with 3.75 ml of methanol-chloroform (2:1, vol/vol) and extracted for 2.5 h at room temperature with intermittent shaking. The suspension was then subjected to sonic treatment in a Bransonic bath for 0.5 h. The membrane residue was removed by centrifugation at 10,000 × *g* for 10 min, and the supernatant was stored at 4°C. The pellet was extracted once more for 1 h after suspension in 4.75 ml of methanol-chloroform-water (2:1:0.8, vol/vol). The suspension was subjected to sonic treatment and centrifuged as described above. The two supernatants were then combined, and 2.5 ml each of chloroform and water were added. The mixture was centrifuged at 2,000 × *g* for 5 min, and the lower, chloroform phase was recovered and then diluted with a small amount of benzene to remove traces of water. The chloroform was removed by evaporation under a stream of nitrogen at 35 to 40°C. The extracted lipids were finally suspended in chloroform-methanol (2:1, vol/vol) and stored at −20°C.

Separation and identification of phospholipids. Phospholipids were separated and analyzed by thin-layer chromatography with Merck Silica Gel H (0.5 mm thick) buffered with 0.5 g of sodium acetate per 100 g of gel (33). Chromatograms were developed in chloroform-methanol-water (65:25:4, vol/vol) or chloroform-acetic acid-methanol-water (80:18:12:5, vol/vol).

The lipid components were characterized by comparing their R_f values with those of known phospholipids and by their reactions with specific spray reagents. Reference lipids were phosphatidylglycerol and diphosphatidylglycerol from *A. laidlawii* A strain EF 22, previously identified by acid and mild alkaline hydrolysis (53). A lipid extract from *E. coli* strain K-12 containing phosphatidylglycerol, phosphatidylethanolamine, and diphosphatidylglycerol (5) was also used. The spray reagents used were: rhodamine 6 G, molybdate reagent for phosphate (10), diphenylamine reagent for lipid sugars (46), ninhydrin (Sigma Chemical Co., St. Louis, Mo.) for amino groups, and periodate-Schiff reagents for α-glycols (43).

Quantitative determination of phospholipids. Phospholipids were separated by thin-layer chromatography as described above, the spots were visualized with iodine vapor, and the individual lipid spots were scraped off into glass tubes. The organic phosphorus assay of McClare (28) was then used. A calibration curve was constructed by applying known amounts of L-α-lysophosphatidyl choline (Sigma) to thin-layer chromatography plates and treating these spots in the same way as the samples.

Isolation of fatty acids. Fatty acids were converted to their methyl esters by treating the lipid extract (0.5 to 1.5 μmol of lipid) for 2 h at 70°C in tubes containing 4 ml of 5% (vol/vol) H_2SO_4 in water-free methanol. The methyl esters were extracted with *n*-hexane (Merck, spectroscopic grade) and analyzed by gas-liquid chromatography.

Identification and quantitative determination of fatty acids. The fatty acids were identified on a Perkin-Elmer model F apparatus equipped with two different columns: (i) a 1.83-m-long glass column, 0.635 cm in diameter, packed with 5% (wt/wt) free fatty acid phase on Chromosorb G AW-DMCS, 80 to 100 mesh, and (ii) a 3.05-m-long stainless steel column, 0.318 cm in diameter, packed with 10% (wt/wt) diethylene glycol succinate on Chromosorb W AW, 80 to 100 mesh. In the former case, the column and injector temperatures were 175 and 300°C, respectively, and the nitrogen flow rate was 25 ml/min. In the latter case, the corresponding parameters were 160 and 330°C and 7 ml/min, respectively. The individual fatty acids were characterized by comparison of retention times with standard mixtures containing methyl esters of straight- and branched-chain C_{13} to C_{20} acids (Larodan Lipids, Malmö, Sweden) run under identical conditions.

Peak areas were calculated with an analog electronic integrator. A known amount of methyl elaidate (Sigma) was added to each sample as an internal standard to obtain absolute values.

RESULTS

Membrane protein composition. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of membrane proteins gave about 35 to 40 well-resolved polypeptide bands (Fig. 1). Most of the bands were found in all strains and over the whole temperature interval investigated, but the relative amounts, as judged from staining intensities, differed for certain proteins. The greatest differences were evident in comparisons between strains (Fig. 1), but small alterations were also noted within each strain when grown at different temperatures (not shown). The two high-molecular-weight proteins (top bands, Fig. 1c) present in the facultatively thermophilic strain Ft R32 were particularly prominent. In the other strains, the 130,000-dalton protein was totally absent, and the 110,000-dalton protein was present in very small amounts. A spectrophotometric scanning of the gel pattern for strain Ft R32 showed that the 130,000-dalton polypeptide band made up 11% and that the 110,000-dalton band made up 28% of the total stain. Thus, roughly 40% of the membrane proteins in strain Ft R32 consisted of these high-molecular-weight proteins. This strain is unique in producing megacin(s) (48, Table 1).

A comparison of the protein patterns between strains Fp 1 and Ot 32 would indicate the magnitude of the difference between the psychrophilic and thermophilic states. About 20 protein bands out of 43 differed in staining intensities when strain Fp 1, grown at 5°C, was compared with strain Ot 32, grown at 70°C (not shown).

Phospholipid composition. Based on comparisons of R_f values and staining behavior with reference lipids on thin-layer chromatography

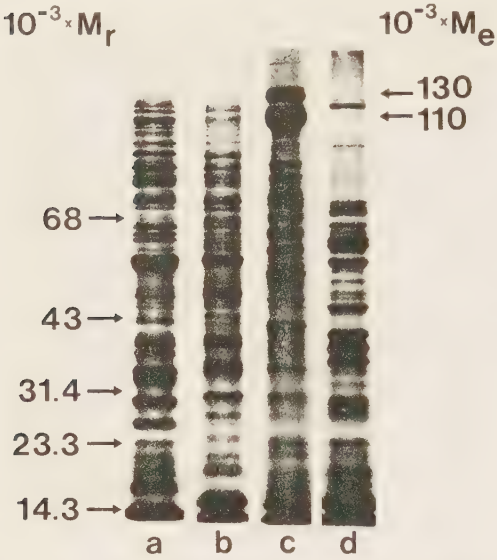

FIG. 1. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of B. megaterium membranes, prepared and solubilized as described in the text. Comparison of protein patterns for strains Fp1, facultative psychrophile (a); M 1, mesophile (b); Ft R32, facultative thermophile (c); and Ot 32, obligate thermophile (d). Each strain was grown close to its optimum temperature (Table 1). The positions of five molecular weight marker proteins are indicated by arrows on the left, and the estimated molecular weights of membrane proteins are indicated on the right.*

plates (24, 53; Materials and Methods), three phospholipids were found in the membranes of all strains and at all growth temperatures. These lipids were phosphatidylethanolamine, phosphatidylglycerol, and diphosphatidylglycerol. Neutral lipids were also present, but these were not further characterized.

Relative changes in phospholipid composition. In the obligate thermophile Ot 32 and the facultative thermophile Ft R32, the dominating phospholipid was phosphatidylethanolamine (Figure 2a and b). The fraction of this lipid increased slightly when the strains were grown at higher temperatures. In both strains, phosphatidylglycerol decreased and diphosphatidylglycerol increased with temperature, although these trends were more pronounced for strain Ft R32.

Diphosphatidylglycerol was the minor phospholipid component in the thermophilic strains with the exception of strain Ft R32 grown at 55°C (Fig. 2a and b). In the mesophile M 1 and the facultative psychrophile Fp 1, however, this phospholipid was the predominant one (Fig. 2c). For the latter strains, changes in phospholipid composition with temperature were not statistically significant. The diphosphatidylglycerol content fluctuated around a mean value of 57% (mol/mol), which was three to six times more than that in the thermophilic strains. The average contents of phosphatidylglycerol and phosphatidylethanolamine were 19 and 23% (mol/mol), respectively.

The lipid-to-protein ratio has been reported to decrease with increasing temperature in membranes of *B. stearothermophilus* (55). This trend can be considered logical, since a higher protein content may render the membrane more rigid and thus help the organism to maintain a functional membrane at higher growth temperatures

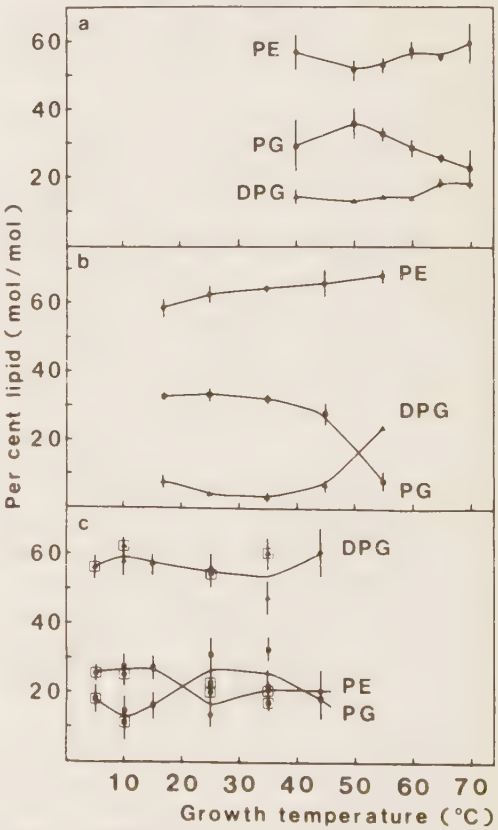

FIG. 2. *Effect of growth temperature on the relative distribution of membrane phospholipids from B. megaterium strains Ot 32, obligate thermophile (a); Ft R32, facultative thermophile (b); and M 1, mesophile/Fp 1 (framed symbols), facultative psychrophile (c). Abbreviations: PE, phosphatidylethanolamine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol. Values reported are the means of four determinations, representing two different cell batches and two analyses of each. Vertical bars indicate standard error.*

(42). However, the phospholipid-to-protein ratio increased over threefold for *B. megaterium* in the presently studied temperature interval (Fig. 3). Similar results have been reported also for the extreme thermophile *Thermus aquaticus*, which is gram-negative but has mainly branched fatty acids (41).

Distribution of fatty acids. The fatty acid methyl esters were analyzed qualitatively by gas-liquid chromatography on two columns with different stationary phases (Materials and Methods). The column containing diethylene glycol succinate gave the best resolution of individual peaks and was further used for the quantitative determinations.

The dominating fatty acids in the membranes of the four *B. megaterium* strains were six branched-chain acids—12-methyltridecanoic (iso-C_{14}), 13-methyltetradecanoic (iso-C_{15}), 14-methylpentadecanoic (iso-C_{16}), 15-methylhexadecanoic (iso-C_{17}), 12-methyltetradecanoic (anteiso-C_{15}), and 14-methylhexadecanoic (anteiso-C_{17}) acids—representing between 60 and 90% (mol/mol) of the total fatty acids, and four straight-chain acids—tetradecanoic (n-C_{14}), pentadecanoic (n-C_{15}), hexadecanoic (n-C_{16}), and octadecanoic (n-C_{18}) acids. In addition, a hexadecenoic acid (n-$C_{\overline{16}}$) was produced in significant amounts by the obligate thermophile Ot 32 when cultivated at temperatures close to the minimum. At 40°C this hexadecenoic acid made up 13% (mol/mol) of the fatty acid total in this strain.

Since the odd-numbered (C_{15} and C_{17}) iso and anteiso acids were the qualitatively predominant ones, only results concerning these acids have been included in the graphs. Figure 4 shows the relative amounts of the branched C_{15}-acids at different growth temperatures. A comparison between strains reveals profound changes in the relative distribution of these acids. The total amount of the acids was approximately constant over the entire temperature interval investigated, but the proportion of iso-C_{15} rose markedly with increased growth temperature. A difference of 41% (mol/mol) was apparent between strain Fp 1 at 5°C and strain Ot 32 at 50°C. In the latter strain, iso-C_{15} then decreased by 15%

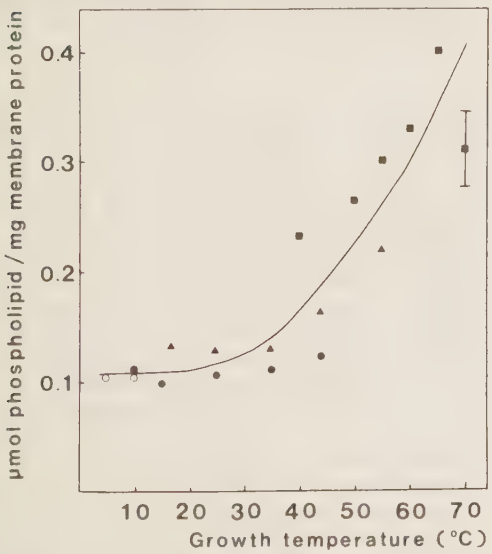

FIG. 3. *Effect of growth temperature on the ratio of micromoles of phospholipid to milligrams of membrane protein in membranes from B. megaterium. Values are shown for strains Fp 1 (○), facultative psychrophile; M 1 (●), mesophile; Ft R32 (▲), facultative thermophile; and Ot 32 (■), obligate thermophile. The curve represents the average trend. Standard error was 8 to 12% of the values (vertical bar).*

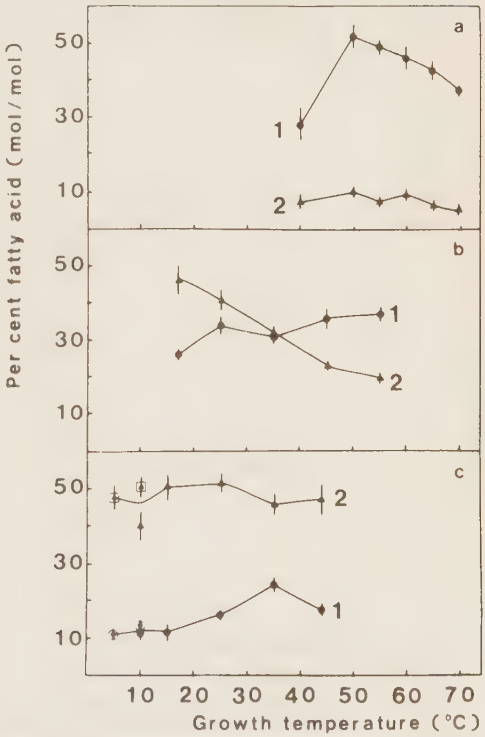

FIG. 4. *Effect of growth temperature on the relative distribution of branched-chain C_{15}-fatty acids (iso [1] and anteiso [2]) in membranes from B. megaterium strains Ot 32, obligate thermophile (a); Ft R 32, facultative thermophile (b); and M 1, mesophile/Fp 1 (framed symbols), facultative psychrophile (c). Values reported are the means of four determinations, representing two different cell batches and two analyses of each. Vertical bars indicate standard error.*

(mol/mol) when the temperature of growth was further raised from 50 to 70°C. Anteiso-C_{15} revealed a 43% (mol/mol) decrease when going from the lower to the higher end point of the temperature interval. The ratios of iso-C_{15} to anteiso-C_{15} increased from 0.23 to 0.40, from 0.56 to 1.87, and from 4.1 to 7.2 for strains Fp 1/M 1, Ft R32, and Ot 32, respectively. Thus, the total rise in this ratio function between 5 and 70°C was over 30-fold. The increase in growth temperature was responsible for a 10-fold rise, whereas the remainder was due to discontinuities between strains.

The trend for iso-C_{17} was similar to that noted for iso-C_{15} (Fig. 4), the increase being 20% (mol/mol) between the end points of the temperature interval (Fig. 5). The relative amount of anteiso-C_{17} also increased with growth temperature when the entire interval was considered, but it decreased within the growth ranges of strains M 1 and Ft R32. In strain Ot 32, the amount of anteiso-C_{17} reached the highest levels. The amount of the branched C_{17}-acids thus increased markedly in this strain, constituting 32% (mol/mol) of the fatty acid total at 70°C. In spite of the same general trend exhibited by these acids, an inversion in the relative amounts was apparent. The ratios of iso-C_{17} to anteiso-C_{17} increased from 0.11 to 0.27, from 0.26 to 3.8, and from 0.86 to 1.81 for strains Fp 1/M 1, Ft R32, and Ot 32, respectively, which corresponds to an over 15-fold rise in this parameter between the end points of the temperature interval.

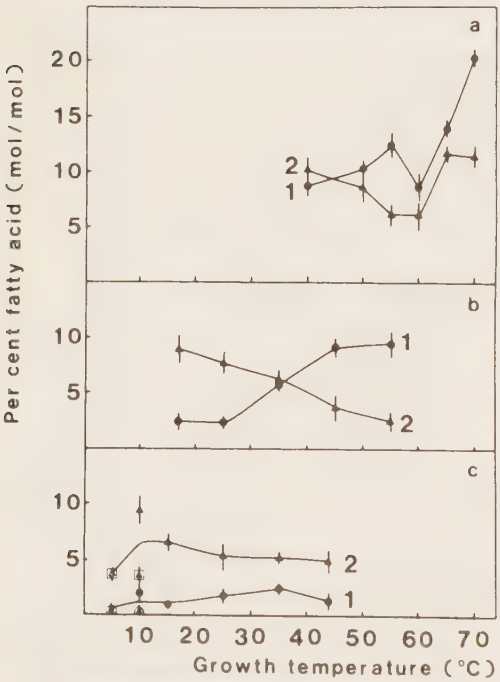

FIG. 5. *Effect of growth temperature on the relative distribution of branched-chain C_{17}-fatty acids (iso [1] and anteiso [2]) in membranes from B. megaterium strains Ot 32, obligate thermophile (a); Ft R32, facultative thermophile (b); and M 1, mesophile/Fp 1 (framed symbols), facultative psychrophile (c). Values reported are the means of four determinations, representing two different cell batches and two analyses of each. Vertical bars indicate standard error.*

The two remaining branched fatty acids, iso-C_{14} and iso-C_{16}, showed minor but consistent trends. The relative amounts of iso-C_{14} decreased from 7% (mol/mol) at 5°C to 1% at 70°C, and iso-C_{16} correspondingly increased from 1% (mol/mol) to 8% between the same temperatures (data not shown).

Evidently, iso acids were synthesized in relatively larger amounts at elevated temperatures (Fig. 4 and 5) than were their anteiso counterparts. Figure 6 shows how the ratio of all iso acids to all anteiso acids depends on growth temperature. A more than 10-fold difference in the value of this parameter between the end points of the investigated temperature interval is evident. This relation thus clearly indicates the important influence of melting points and the packing properties of the respective fatty acids in the functional adaptation of the membrane composition to environmental temperature.

Elongation of the acyl chains increases the fatty acid melting point (49). The fatty acids of the present *B. megaterium* strains may be classified into long- and short-chain groups. The former group includes all saturated C_{16}-, C_{17}-, and C_{18}-acids, and the latter includes all saturated C_{14}- and C_{15}-acids. There was about a fivefold increase of long-chain fatty acids over short-chain ones in the present temperature interval (Fig. 7). The ratios of iso-C_{17}/iso-C_{15}, anteiso-C_{17}/anteiso-C_{15}, and iso-C_{16}/iso-C_{14} increased from 0.04 to 0.55, from 0.08 to 2.2, and from 0.14 to 8.5, respectively, between 5 and 70°C.

DISCUSSION

The results presented have centered on only one aspect of temperature adaptation in these *B. megaterium* variants. Apart from changes in membrane composition, there is a genetic aspect to temperature adaptation. It was suggested (48) from data on the origin and reversion of these variants that plasmids could be involved in the phenomenon. The two high-molecular-weight proteins in strain Ft R32 (Fig. 1c) could be

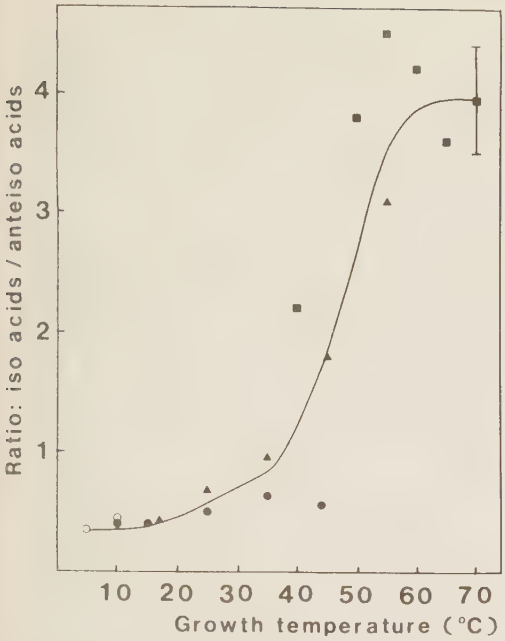

FIG. 6. *Effect of growth temperature on the ratio between relative amounts of iso and anteiso fatty acids in membranes from B. megaterium. Iso fatty acids represent the sum of iso-C_{14}, iso-C_{15}, iso-C_{16}, and iso-C_{17}, and anteiso fatty acids represent the sum of anteiso-C_{15} and anteiso-C_{17}. Values are shown for strains Fp 1 (○), facultative psychrophile; M 1 (●), mesophile; Ft R32 (▲), facultative thermophile; and Ot 32 (■), obligate thermophile. The curve represents the average trend. Standard error, calculated by propagation of error, was 7 to 10% of the values (vertical bar).*

plasmid determined. Iyer (17) reported substantial variations in the amounts of two proteins (34,000 and 36,500 daltons) located in the outer membrane of *E. coli* B/r when the organism harbored different antibiotic resistance plasmids. Similarly, a 24,000-dalton protein was found in membrane preparations from strains of *E. coli* K-12 carrying the F-factor and the derepressed plasmids R100-1 and R1-19 (32). More detailed comparisons are premature, since, to our knowledge, no information is available concerning membrane protein patterns in thermophilic bacteria.

The distribution of fatty acids in the mesophilic wild type M 1 corresponds to that reported for *B. megaterium* (19). The mesophilic *Bacillus* species may be divided into two groups based on their respective fatty acid compositions: one producing a high proportion of anteiso acids, with anteiso-C_{15} dominating, and the other producing odd-numbered iso acids, especially iso-C_{15}, in the largest amount (20). *B. megaterium* is usually included in the former group (23), which is even clearly possible for strain M 1 (Fig. 4c and 5c). However, in the obligate thermophile Ot 32, the odd-numbered iso acids are predominant (Fig. 4a and 5a), relating this strain to the second group. The transition in group relations occurs within the growth range of the facultative thermophile Ft R32. When grown below 35°C, it belongs to group one, but at temperatures above 35°C, it belongs to group two (Fig. 4b and 5b). The validity of such a classification may therefore be questioned. The thermophilic strains at high temperatures resemble *B. stearothermophilus* (8, 47, 56), *B. caldolyticus*, and *B. caldotenax* (52) in producing mainly odd-numbered iso acids.

The same increasing trend from the lower to the higher end point of the temperature interval was apparent for the ratios of both iso-C_{15}/anteiso-C_{15} and iso-C_{17}/anteiso-C_{17} (Fig. 4, 5). This trend appears logical when viewed in light of the different physical properties of the

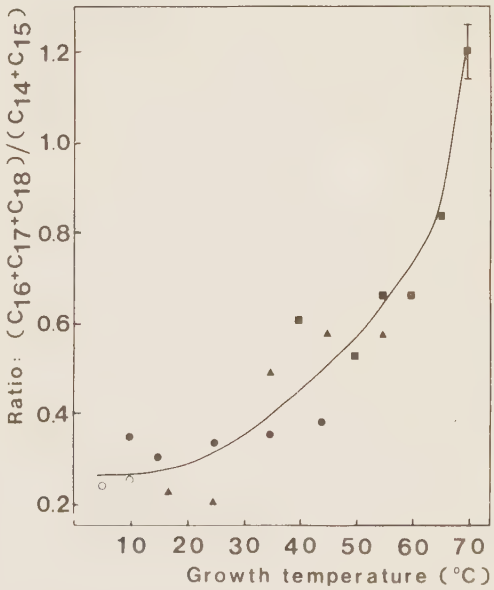

FIG. 7. *Effect of growth temperature on the ratio between relative amounts of long-chain and short-chain fatty acids in membranes from B. megaterium. Long-chain acids are represented by the sum of iso-C_{16}, n-C_{16}, iso-C_{17}, anteiso-C_{17}, and n-C_{18}, and short chain acids are represented by the sum of iso-C_{14}, n-C_{14}, iso-C_{15}, anteiso-C_{15}, and n-C_{15}. Values are shown for strains Fp 1 (○), facultative psychrophile; M 1 (●), mesophile; Ft R32 (▲), facultative thermophile; and Ot 32 (■), obligate thermophile. The curve represents the average trend. Standard error, calculated by propagation of error, was 5 to 8% of the values (vertical bar).*

fatty acids involved. The iso-C_{15}- and iso-C_{17}-acids have melting points similar to those of their straight-chain isomers, but their lateral packing areas are at least 1.5 times larger (54). The anteiso acids, on the other hand, have significantly lower melting points (about 25°C) than the corresponding straight-chain acids and occupy still larger lateral packing areas (54). At higher growth temperatures, the incorporation of the iso acids would be preferred to that of the anteiso acids, thus preventing the membrane from becoming too fluid and leaky at the higher temperatures. A deviation from the general tendency of increased amounts of iso-C_{15}-acids at higher growth temperatures was noted in strain Ot 32 between 50 and 70°C. Iso-C_{15} was, however, replaced primarily by iso-C_{17} and, to a smaller extent, by n-C_{16} and n-C_{18} in this interval, all acids having higher melting points than iso-C_{15}.

A decrease in the total amount of branched fatty acids with increasing growth temperature has been reported in several investigations of *Bacillus* species (4, 8, 44, 47, 52, 56). This trend was most obvious in *B. stearothermophilus* (8, 47, 56) and was, in this species, accompanied by a drastic increase in the amount of n-C_{16}. In strain Ot 32 grown between 50 and 70°C and in strain Ft R32, the total amounts of branched fatty acids decreased 8 and 15% (mol/mol), respectively, with concomitant increases in the amounts of n-C_{16} and n-C_{18}. However, the changes between branched-chain and straight-chain acids were neither the dominant ones nor the ones solely responsible for the observed alterations in membrane fatty acid composition with growth temperature. Instead, the changes between the two groups of branched-chain fatty acids, iso and anteiso, were of much greater magnitude in all strains. The high-melting iso acids seem to play the role of saturated acids and the low-melting anteiso acids seem to play that of unsaturated acids in their abilities to regulate membrane fluidity, when *B. megaterium* is compared to an organism synthesizing exclusively straight-chain acids. As seen in Fig. 4 through 6, each strain has the capacity to alter the ratio of iso acids to anteiso acids within certain limits, but this alteration is carried out at different ratios in the different strains. At a growth temperature common to all strains, for instance 40°C, the iso-C_{15}/anteiso-C_{15} ratios were 0.46, 1.26, and 4.1 for strains M 1, Ft R32, and Ot 32, respectively.

The synthesis of unsaturated acids by the genus *Bacillus* has been reported for *B. stearothermophilus* (8, 15, 56) and for a number of mesophilic and psychrophilic species (15, 21, 22). In strain Ot 32, the hexadecenoic acid together with n-C_{16} and iso-C_{16} replaced iso-C_{15} at 40°C.

When the hydrocarbon chain is elongated within a homologous series of fatty acids, the acids acquire higher melting points because of the additive London-van der Waals forces (49). The elongation process is thus another way to solidify the membrane lipids in *B. megaterium* at higher growth temperatures (Fig. 7). The phenomenon has been observed in other investigations as well (38, 44, 52).

The finding of phosphatidylethanolamine, phosphatidylglycerol, and diphosphatidylglycerol in the *B. megaterium* membranes is in agreement with earlier observations (2, 36, 37). Neither glycosaminyl nor lysyl derivatives of phosphatidylglycerol were synthesized under the presently used growth conditions. The pH of the culture during growth was between 6.5 and 7.5, and the glycosaminyl derivative is formed mainly at a pH around 5 (2, 36, 37).

The biosyntheses of phosphatidylethanolamine, on one hand, and phosphatidylglycerol and diphosphatidylglycerol, on the other, proceed along different pathways from the common precursor, CDP-diglyceride. These pathways have been established for *E. coli* as well as for some *Bacillus* species (14, 26, 40). In *E. coli*, the ratio between the relative amounts of phosphatidylethanolamine and phosphatidylglycerol/diphosphatidylglycerol is fairly constant in the temperature range of the organisms (5, 9, 45). We found small variations in this ratio within each *B. megaterium* strain and a drastic inversion when going from the mesophilic to the thermophilic state (Fig. 2). The different chemical and physical properties of the phospholipids may partly explain these results. There were no differences in fatty acid content in the individual phospholipids (unpublished results). The biophysical behavior of the lipids as determined by the polar headgroup is therefore of main interest. Phosphatidylethanolamine is known to have its phase transition temperature well above that of phosphatidylglycerol when the fatty acid content is identical in both lipids (6, 51). Phosphatidylethanolamine can accordingly be more densely packed, and an increased relative amount of this lipid in the membranes at higher growth temperatures would be favorable in maintaining membrane integrity. A similar trend has been noted in *Tetrahymena pyriformis* (31). Moreover, a sixfold increase in the ratio of phosphatidylethanolamine to phosphatidylcholine in membranes from mouse LM cells was accompanied by a markedly raised viscosity (12).

An additional explanation of the reversed phospholipid pattern in strains Fp 1 and M 1 as compared with the thermophilic strains is possible. Several respiratory chain enzymes require

primarily diphosphatidylglycerol to attain full activity (7, 11). The thermophilic strains are more anaerobic than the mesophilic ones (48). When grown under the same conditions of aeration, the latter strains probably synthesize greater amounts of the respiratory chain components and accordingly need more diphosphatidylglycerol. Op den Kamp et al. (37) cultivated *B. megaterium* MK 10D under strong aeration and found that this lipid made up only 5% (wt/wt) of the phospholipid total. It has been suggested that diphosphatidylglycerol contributes to the thermal stability of *B. stearothermophilus* membranes (34). However, in our *B. megaterium* strains, this function of the lipid is less probable, since it was the minor membrane phospholipid component in the thermophilic-strains (Fig. 2).

It should be pointed out that profound changes in the investigated parameters occurred in the facultatively thermophilic strain Ft R32 between 35 and 50°C (Fig. 3 through 7). This temperature range corresponds to the boundary between bacterial mesophily and thermophily established for *B. licheniformis* (1).

The regulation of membrane lipid properties occurring in *B. stearothermophilus*, *B. caldolyticus*, and *B. caldotenax* (8, 47, 52, 56) cannot be considered as specific for thermophilic species, since a similar regulation mechanism is found in a thermophilic variant of an otherwise mesophilic *B. megaterium*. The genetic information for adaptation of the cell membrane composition to more or less extended thermophilic growth may thus be present in the *B. megaterium* wild-type population but is not expressed in this population under mesophilic growth conditions.

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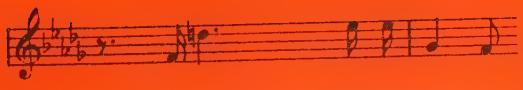

Cubic liquid crystalline phase with phosphatidylethanolamine from *Bacillus megaterium* containing branched acyl chains.

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1. Introduction

A large number of organisms, procaryotic as well as eucaryotic, respond to variations in the environmental temperature by changing the acyl chain composition of the membrane lipids [1-6]. The most emphasized regulation mechanism is by far the increase in acyl chain unsaturation when the temperature is decreased. Alternative regulation mechanisms are expected to operate in organisms synthesizing small amounts of unsaturated fatty acids and large amounts of branched-chain fatty acids. This was found in investigations of the acyl chain composition of Bacillus megaterium membranes [7]: the ratio iso/anteiso acyl chains is decreased and the hydrocarbon chain length is reduced when the growth temperature is lowered.

The basic structure of all biological membranes is the lipid bilayer matrix. In spite of this, most membranes contain at least one lipid that forms a non-lamellar phase structure with water. Phosphatidylethanolamine (PE) [8,9], phosphatidylserine [10], monogalactosyldiglyceride [11] and monoglucosyldiglyceride [12] can form a reversed hexagonal (H_{II}) liquid crystalline phase in equilibrium with a free water phase. The transition from a lamellar to a non-lamellar phase can be triggered by several environmental factors such as temperature, pH and concentration of ions [8-10,13,14]. Even if a transition to a non-lamellar phase does not occur, the temperature affects the packing of lipid molecules [3]. An organism is consequently often forced to regulate the membrane lipid composition in response to changes in growth temperature in order to avoid disturbances in membrane functions [3].

In this report we show that PE isolated from B. megaterium grown at 20°C forms a lamellar liquid crystalline phase at the growth temperature, and at low water contents an isotropic (cubic) liquid crystalline phase at 58°C. This lipid has a low ratio iso/anteiso acyl chains. PE isolated from this organism grown at 55°C forms only a lamellar liquid crystalline phase up to at least 65°C. The ratio iso/anteiso acyl chains is ten times higher for this lipid.

2. Experimental

A facultatively thermophilic strain (Ft R32) and an obligately thermophilic strain (Ot 32) of Bacillus megaterium were grown at 20°C and 55°C, respectively, in tryptone starch broth [7]. The temperature ranges of the strains were tested before the culture vessel was inoculated. Cells were harvested by centrifugation for 20 min at 8000xg, and cell membranes were prepared as described in [7]. Lipids were extracted from the dried membranes with chloroform-methanol (2:1, v/v), methanol-chloroform (2:1, v/v), and methanol. During the extractions the membrane suspensions were sonicated in a Bransonic bath for 30 minutes. The membrane residues were collected on a filter paper and non-lipid contaminants in the extract were removed by passage through a Sephadex® G25 Fine (Pharmacia) column [15]. PE was isolated by thin-layer chromatography (TLC) on Silica gel 60H (Merck) [7]. The purity of the lipid preparations was over 95% as judged by TLC. The lipids were stored as concentrated solutions in chloroform-methanol (2:1, v/v) at -70°C. The acyl chain composition of the lipids was determined by gas-liquid chromatography (GLC) on a diethylene glycol succinate column operating at 160°C [7].

Samples for nuclear magnetic resonance (NMR) studies were prepared as described in [16,17]. The heavy water concentrations were chosen both above and below the maximum hydration capacity of PE. ^{2}H NMR spectra were recorded at 15.35 MHz on a modified Varian XL-100-15, and analyzed as described in [12,17,18]. ^{31}P NMR measurements were taken at 101.27 MHz on a Bruker WM-250 NMR spectrometer equipped with a superconducting magnet. To remove the dipolar interaction between the phosphorus and the protons, the latter were irradiated with an intense decoupling field. The samples were thermally equilibrated within

$1^{o}C$ for at least one hour before the spectra were taken. After the recording of the spectra the samples were analyzed for lipid degradation by TLC and GLC. No degradation products were found. The lipid samples were also studied between two crossed polarizers and by polarized light microscopy. Each phase shows a typical microscopic texture [19].

The translational diffusion coefficient of PE in the cubic phase was measured with the NMR diffusion method described elsewhere (see e.g. [16]).

3. Results

The acyl chain composition of the two different PE preparations is shown in Table 1. Anteiso-C_{15} was the dominating acyl chain in PE isolated from Ft R32. The ratio iso/anteiso was very low, and the C_{15} chains made up nearly 85 mol% of the total amount of acyl chains. In Ot 32 the ratio iso/anteiso was increased by 10-fold as compared to Ft R32, and the chain length parameter was about eight times higher.

PE isolated from Ft R32 formed a lamellar liquid crystalline phase with water within the range of 3 to 14 moles of ^2H_2O per mol of lipid (Table 2). The samples were optically birefringent, exhibited a fine mosaic microscopic texture [19], and showed a ^2H NMR quadrupole splitting (Figure 1d) [16,17]. ^{31}P NMR spectra exhibited chemical shielding anisotropy of the order of -40 ppm with a high field peak and a low field shoulder (Figure 1a), indicating a lamellar liquid crystalline phase [20,21]. The lamellar phase was stable up to 50°C with 3 moles of ^2H_2O per mol of lipid (Table 2). Above this temperature the sample consisted of two liquid crystalline phases, one lamellar and one viscous isotropic (cubic) phase. This was evident from the ^{31}P and ^2H NMR spectra, both of which revealed an anisotropic part together with a central isotropic peak (Figure 1b,e) [16,17]. Ocular inspection of the sample between two crossed polarizers showed an anisotropic phase along with an optically isotropic phase. Above 57°C a pure cubic liquid crystalline phase (one-phase system) was formed with 3 moles of ^2H_2O per mol of lipid (Figure 1c,f). The transition temperature for the lamellar phase increased by increasing the water content of the samples, and with 12,5 moles of ^2H_2O per mol of lipid the cubic phase began to appear at 63°C (Table 2). A preliminary study with polarizing light microscopy of the sample with 14 moles of ^2H_2O per mol of lipid

revealed that the transition between the lamellar and cubic phases began at about 70°C. A pure cubic phase formed at about 90°C, as shown by the dark microscopic background. Above 105°C a shiny angular texture, typical for hexagonal liquid crystalline phases [19], began to appear. The cubic phase gradually transformed into the hexagonal (probably H_{II}) phase between 105 and 115°C. When PE isolated from Ft R32 was mixed with more than 14 moles of ^2H_2O per mol of lipid, two-phase systems were formed, consisting of a lamellar phase and a free water phase. The lamellar phase was stable up to at least 65°C. The free water phase was evident from a central peak with very narrow line width in the ^2H NMR spectra.

PE isolated from Ot 32 formed a lamellar liquid crystalline phase with water between 26 and 65°C, both below and above the maximum hydration capacity of the lipid (Table 3). NMR spectra of the type shown in Figure 1a,d was obtained, and the samples were optically birefringent. A two-phase system consisting of a lamellar phase and a free water phase was achieved when the lipid samples contained more than 8 moles of ^2H_2O per mol of lipid.

In a preliminary investigation of the diffusion coefficient of PE in the cubic phase at 65°C, it was found that $D_L \approx 5 \cdot 10^{-12}\ m^2 \cdot s^{-1}$. This value is of the same order of magnitude as the lateral diffusion coefficient observed for lipids in bilayers, strongly indicating that the cubic phase is bicontinuous and built up of continuous hydrocarbon regions [16,29].

4. Discussion

It is shown that the phase equilibria in the PE/water system are influenced by changes in the ratio iso/anteiso acyl chains of the lipid molecules. The phase equilibria for PE also depend on the degree of unsaturation of straight acyl chains [20]. As demonstrated previously [3,22] the geometry of the lipid molecules is of particular importance for determination of the lipid aggregate shape. Three quantities can be used to describe the shape of lipid molecules: the hydrocarbon chain volume ($\underline{v}$), the hydrocarbon-water interfacial area ($\underline{a}$) and the hydrocarbon chain length ($\underline{l}$). When the packing parameter $\underline{v}(\underline{a} \cdot \underline{l})^{-1} =$ 1/2-1 the bilayer is the preferred structure. A non-lamellar structure composed of long water rods embedded in a hydrocarbon matrix ("water-in-oil" aggregates) may form if the packing parameter exceeds unity. An increase in the degree of unsaturation of the acyl chains results in a higher value of $\underline{v}(\underline{a} \cdot \underline{l})^{-1}$ for a lipid molecule [3,22]. It can therefore be predicted that the most unsaturated PE:s should form an H_{II} phase while the more saturated species should form a lamellar phase at a certain temperature. This has also been found experimentally [20].

Iso acids have their branching point at the penultimate carbon atom while anteiso acids have this point displaced one carbon atom towards the carboxyl group. Anteiso acids can accordingly not be as closely packed as iso acids. This is supported by experimental observations: (1) anteiso acids occupy a larger area than iso acids in a monolayer [23]; (2) anteiso acids have lower melting points than the corresponding iso acids [24]; synthetic phosphatidylcholines containing anteiso acyl chains have considerably lower gel to liquid crystalline transition temperatures than the corresponding iso derivatives [25,26]. When the

ratio iso/anteiso acyl chains is decreased the effective hydrophobic volume ($\underline{v}$) of PE is probably increased. The packing parameter $\underline{v}(\underline{a} \cdot \underline{l})^{-1}$ assumes a higher value and PE isolated from strain Ft R32 can be postulated to have an increased tendency to form cubic and H_{II} phases as compared to PE isolated from strain Ot 32. This was actually found (Tables 2,3). The present results further support the conclusion made earlier [7] that iso and anteiso acyl chains play the roles of straight saturated and unsaturated acyl chains, respectively, in a membrane containing large amounts of these components. However, the difference in physical properties between iso and anteiso acyl chains is less than the difference between straight saturated and cis-unsaturated acyl chains [25,26]. Dioleoylphosphatidylethanolamine forms an H_{II} phase in excess water already at 15°C [20].

The capacity to take up water is different for the two PE preparations (Tables 2,3). This reflects the different packing properties of iso and anteiso acids. Since the area occupied by the phosphorylethanolamine group is smaller than that occupied by the two acyl chains [27], an increase in the width of the hydrophobic part of PE results in an increased distance between the polar head groups. PE isolated from strain Ft R32, with a large amount of the more loosely packed anteiso acyl chains, is accordingly able to take up larger amounts of water.

Some biological membrane lipids have earlier been found to form cubic phases together with water. These phases are formed either by single lipids such as lecithin [28,29], PE with straight acyl chains at a very low pH [9] and dioleoylmonoglucosyldiglyceride [30], or by lipid mixtures such as monogalactosyldiglyceride/digalactosyldiglyceride [31] and monoglucosyldiglyceride/diglucosyldiglyceride [16,17].

Although both the PE preparations used in this work form a lamellar phase in excess water up to at least 65°C, they have quite different packing properties. It has been shown that the permeability barrier of the bilayer is destroyed before the lamellar phase is transformed into micelles or an H_{II} phase [32,33]. Moreover, in Escherichia coli membranes the increase in permeability as a result of enhanced growth temperature is counteracted by reducing the degree of unsaturation of the phospholipid acyl chains [34]. Since an increase in temperature will increase the tendency of PE to form cubic and H_{II} phases, the temperature-induced regulation of acyl chain composition in B. megaterium membranes [7] is most probably necessary in order to maintain an optimal packing of the lipid molecules. Similar regulation mechanisms, related to the phase structures formed by membrane lipids, have been found to operate in Pseudomonas fluorescens [35] and Acholeplasma laidlawii [3,36]. In this report PE isolated from two different strains of B. megaterium was used. However, the same qualitative difference in acyl chain composition as noticed between the strains is obtained within the growth temperature interval of Ft R32 [7]. Furthermore, a shift in growth temperature when this strain is in the early logarithmic phase of growth elicits a rapid and profound analogous change in acyl chain composition [T. Clementz and L. Rilfors, unpublished results], indicating that the change is vital for proper membrane function. The regulation of the ratio iso/anteiso acyl chains in the membrane lipids is relevant not only to the genus Bacillus. Several other procaryotes, e.g. many extreme thermophiles, contain lipids with mainly branched acyl chains [24]. A temperature dependent alteration of the ratio iso/anteiso acyl chains like the one found for B. megaterium [7] has also been reported [24].

Acknowledgements

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Table 1. Acyl chain composition in PE isolated from *Bacillus megaterium* strain Ft R32 grown at 20°C and strain Ot 32 grown at 55°C. The values are given as mol%.

Acyl chain	Ft R32 20°C	Ot 32 55°C
Iso-C_{14}	0.9	0.3
Normal-C_{14}	0.1	0.5
Iso-C_{15}	18.2	35.8
Anteiso-C_{15}	65.3	4.7
Normal-C_{15}	-	0.3
Iso-C_{16}	0.9	6.6
Normal-C_{16}	0.2	3.7
Iso-C_{17}	0.6	28.5
Anteiso-C_{17}	2.7	17.6
Normal-C_{17}	-	2.1
Unidentified acyl chains (C_{16}-C_{18})	11.2	-
Σ normal acyl chains	0.3	6.6
Σ branched acyl chains	88.5	93.4
Acyl chain ratios		
$\frac{\Sigma \text{ iso}}{\Sigma \text{ anteiso}}$	0.30	3.19
$\frac{\Sigma\ C_{16}\text{-}C_{18}}{\Sigma\ C_{14}\text{-}C_{15}}$	0.18	1.41

Table 2. Quadrupole splittings (Δ^2H) and phases obtained for different PE/water mixtures. PE was isolated from *Bacillus megaterium* strain Ft R32 grown at 20°C.

Moles of ^2H_2O per mol of lipid	Temperature (°C)	Δ^2H (kHz)	Phase(s)
3.0	26	1.6	Lamellar
	45	1.4	Lamellar
	50	1.4	Lamellar + cubic
	54	1.4	Lamellar + cubic
	58	-	Cubic
4.5	26	2.2	Lamellar
	47	1.8	Lamellar
	50	2.0	Lamellar + cubic
	57	-	Cubic
	64	-	Cubic
6.0	26	1.6	Lamellar
	53	2.0	Lamellar
	58	1.8	Lamellar + cubic
	65	-	Cubic
11.0	26	0.9	Lamellar
	55	1.1	Lamellar
	58	1.1	Lamellar + cubic
	60	1.2	Lamellar + cubic
	66	-	Cubic
12.5	26	0.8	Lamellar
	60	1.0	Lamellar
	63	1.1	Lamellar + cubic
14.0	26	0.8	Lamellar
	65	1.0	Lamellar
15.0	26	0.8	Lamellar + free water
	65	0.9	Lamellar + free water
19.0	26	0.6	Lamellar + free water
	65	0.7	Lamellar + free water

Table 3. Quadrupole splittings (Δ^2H) and phases obtained for different PE/water mixtures. PE was isolated from *Bacillus megaterium* strain Ot 32 grown at 55°C.

Moles of ^2H_2O per mol of lipid	Temperature (°C)	Δ^2H (kHz)	Phase(s)
3.0	26	1.4	Lamellar
	65	1.6	Lamellar
4.5	26	1.6	Lamellar
	47	2.6	Lamellar
	64	2.7	Lamellar
7.0	26	1.2	Lamellar
	62	2.2	Lamellar
8.5	26	1.1	Lamellar + free water
	64	2.1	Lamellar + free water
10.0	26	1.1	Lamellar + free water
	65	2.1	Lamellar + free water
11.5	26	1.1	Lamellar + free water
	51	1.4	Lamellar + free water
	65	1.7	Lamellar + free water

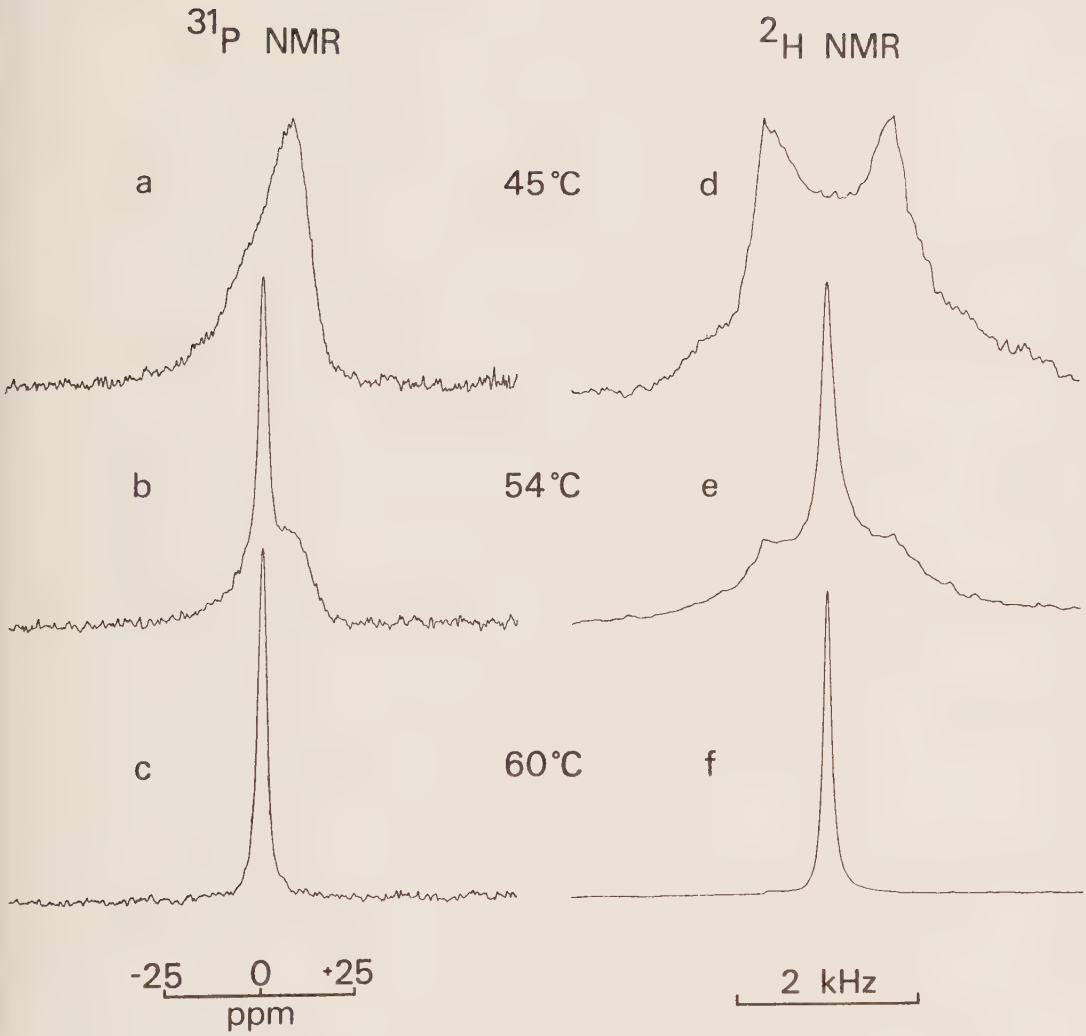

Figure 1. ^{31}P NMR (a-c) and ^{2}H NMR (d-f) spectra of PE isolated from Bacillus megaterium strain Ft R32 grown at 20°C. The water content was 3 moles of $^{2}H_2O$ per mol of lipid. (a,d) lamellar liquid crystalline phase; (b,e) two-phase sample consisting of lamellar + cubic liquid crystalline phases; (c,f) cubic liquid crystalline phase.

VI

Membrane lipid composition in *Acholeplasma laidlawii* A grown on iso and anteiso branched-chain saturated fatty acids.

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Abbreviations: $Acyl_2GlcGro$, diacylmonoglucosylglycerol; $Acyl_2Glc_2Gro$, diacyldiglucosylglycerol; PtdGro, phosphatidylglycerol; PtdEtn, phosphatidylethanolamine; GLX, glucolipid X; GLC, gas-liquid chromatography; TLC, thin-layer chromatography.

SUMMARY

1. Growth of Acholeplasma laidlawii strain A is supported by the saturated branched-chain fatty acids 2-methylbutanoic acid (anteiso-C_5), 12-methyltetradecanoic acid (anteiso-C_{15}), 14-methylhexadecanoic acid (anteiso-C_{17}), 3-methylbutanoic acid (iso-C_5) and 13-methyltetradecanoic acid (iso-C_{15}). These fatty acids are thus able to replace the growth requirement of this strain for an exogenous long-chain unsaturated or cyclopropane fatty acid. The difference between A. laidlawii strains A and B, as regards their fatty acid growth requirements, appears to be minimal.

2. The polar head group composition of the A. laidlawii A membrane lipids is dependent on the type of branched-chain fatty acid incorporated into the membrane. The ratio between the two dominating lipids, diacylmonoglucosylglycerol and diacyldiglucosylglycerol, is increased when anteiso acyl chains are replaced by iso acyl chains. The growth temperature and incorporation of cholesterol also affects the polar head group composition.

3. The average acyl chain length and the ratio iso/anteiso acyl chains are reduced when the growth temperature is lowered. Shorter acyl chains are synthesized when A. laidlawii A is grown on iso-C_5 as compared to anteiso-C_5.

4. In A. laidlawii A membranes, alterations in the incorporated ratios of iso/anteiso acyl chains and straight saturated/cis-unsaturated acyl chains yield the same qualitative respons in polar head group composition. The steric configuration of the acyl chains, and thus the effective shape of the lipid molecules, governs the regulation of lipid composition. This is supported by the finding that phosphatidylethanolamine containing a low ratio iso/anteiso acyl chains forms a

cubic phase, while the lipid forms a lamellar phase with a high ratio iso/anteiso acyl chains [Rilfors *et al*. (1982) FEBS Lett., in press].

INTRODUCTION

Several genera of bacteria have membrane lipids which contain mainly iso and anteiso methyl-branched saturated acyl chains [1]. Of these genera, Bacillus has been most thoroughly studied. The two kinds of fatty acids have different physico-chemical properties: anteiso fatty acids have lower melting points [1] and occupy larger areas in a monolayer at the water-air interface [2], than do iso fatty acids of the same chain length. In membrane lipids of Bacillus megaterium the ratio iso/anteiso acyl chains and the hydrocarbon chain length are reduced by lowering the growth temperature [3,4].

Acholeplasma laidlawii A regulates the polar head group composition of the membrane lipids upon: (1) incorporation of different straight-chain saturated and unsaturated fatty acids; (2) changes in growth temperature; and (3) incorporation of cholesterol and anesthetics into the membrane [5-9]. The regulation of the lipid composition has been interpreted as an effort to maintain optimal packing of the bilayer structure [10,11], and the discussions are based on recent developments of the theory of self-assembly of amphiphiles [12,13]. The validity of this interpretation is supported by investigations of the phase structures formed by mixtures of the two dominating lipids in the A. laidlawii membrane, diacylmonoglucosylglycerol ($Acyl_2GlcGro$) and diacyldiglucosylglycerol ($Acyl_2Glc_2Gro$) [14,15]. The phase equilibria in glucolipid-water systems are affected by: (1) the ratio $Acyl_2GlcGro$/ $Acyl_2Glc_2Gro$; (2) the degree of acyl chain unsaturation; (3) temperature; and (4) the presence of cholesterol. It has also been shown that the phase equilibria in phosphatidylethanolamine (PtdEtn)-water systems are influenced by changes in the ratio iso/anteiso acyl chains in this lipid [4]. It can therefore be expected that the polar head group

composition in A. laidlawii A membranes is altered by incorporation of different types of branched-chain fatty acids.

A. laidlawii strain B has been shown to incorporate long branched-chain fatty acids [16-19] as well as branched-chain fatty acid precursors [20] into the membrane lipids. To my knowledge no report on the growth of A. laidlawii strain A on these fatty acids exists. It has been asserted that strain A, in contrast to strain B, is dependent on a long-chain unsaturated or cyclopropane fatty acid for growth [21-24].

The aim of this study was: (1) to lend further support for our earlier statement that regulation of lipid composition in A. laidlawii A is governed by the effective shape of the lipid molecules [10,11]; (2) to make a detailed investigation of the membrane lipid composition of A. laidlawii A when the organism is grown on iso and anteiso methyl-branched fatty acids; (3) to investigate if the temperature-induced changes in the branched acyl chain composition of B. megaterium membrane lipids [3] occur in other biological membranes as well, and thus reflect the physico-chemical properties of the branched acyl chains; and (4) to compare the fatty acid growth requirements of A. laidlawii strains A and B.

MATERIALS AND METHODS

Organisms and growth conditions

A. laidlawii strain A (EF22) [5] was used in all experiments. Furthermore, *A. laidlawii* strain B [17], kindly provided by Dr. R.N. McElhaney, was used in the fatty acid growth requirement studies. The organisms were grown in a lipid-depleted bovine serum albumin/tryptose medium prepared as described previously [6,8]. The medium was supplemented with fatty acids in ethanolic solutions: (a) 120 μM of one of 12-methyltetradecanoic (anteiso-C_{15}), 14-methylhexadecanoic (anteiso-C_{17}), 13-methyltetradecanoic (iso-C_{15}), 15-methylhexadecanoic (iso-C_{17}), dodecanoic (n-C_{12}), tetradecanoic (n-C_{14}), pentadecanoic (n-C_{15}), hexadecanoic (n-C_{16}), heptadecanoic (n-C_{17}) or octadecanoic (n-C_{18}) acids; (b) different equimolar mixtures of anteiso-C_{15}, anteiso-C_{17}, iso-C_{15} and iso-C_{17}, the total concentration being 120 μM; and (c) 12 μM of pantetheine together with 1.0 mM of 2-methylbutanoic acid (anteiso-C_5), 3-methylbutanoic acid (iso-C_5), or a mixture of these acids (0.5 mM each). Cholesterol was added to a final concentration of 20 μM. Incorporation of cholesterol into the membranes was followed by adding 30 μCi/l of [^{3}H] cholesterol (40 Ci/mmol). The final concentration of ethanol in the medium was 0.5% (v/v) or less, which does not impair cell growth.

Cells were cultured at 27 and 37°C, and harvested in the late logarithmic or early stationary phase of growth 16 to 22 h after inoculation. The organism was subcultured at least five times in medium with each type of supplementation and at the proper temperature before the final cultivation. Cell growth was monitored by measuring the optical density at 540 nm (OD_{540}).

Membrane preparation and lipid extraction

Cells were harvested and membranes were prepared as described earlier [6]. Each membrane preparation was extracted with 14 ml of chloroform-methanol (2:1, v/v) for 1 h at room temperature, 0.7 ml of double destilled water was added, and the lipid extracts were purified from non-lipid contaminants by passage through a Sephadex® G25 Fine column [25] . The solvents were evaporated at 37°C under a stream of nitrogen. The lipids were finally dissolved in a small volume of chloroform-methanol (2:1, v/v), and stored at -20°C until performance of the analyses.

Determination of polar head group and acyl chain composition

In the experiments with the long branched-chain fatty acids the different lipids were quantified by gas-liquid chromatography (GLC) after separation by thin-layer chromatography (TLC) [5,6]. Before the TLC-analysis, each lipid extract derived from these experiments was mixed with an A. laidlawii A reference membrane lipid extract containing ^{14}C-labelled cis,cis-9,12-octadecadienoyl (linoleoyl) chains. Avidin was used to stop the de novo fatty acid synthesis [18] when A. laidlawii was grown on linoleic acid, and the linoleoyl chains constituted 98 mol% of the acyl chains in the reference extract. The lipid spots were visualized with rhodamine 6G, scraped into test tubes, and the silica gel was dried overnight at a pressure of 0.1 mm Hg. Four ml of water-free methanol containing 5% (v/v) H_2SO_4 was added to each tube, and the acyl chains were methylated at 70°C for 2 h. The methyl esters were extracted with n-hexane after addition of 2 ml of water to each tube. This procedure results in a quantitative recovery of the acyl chains. The solvent was evaporated at 30°C under a stream of nitrogen and the

methyl esters were dissolved in 100 µl of n-hexane. The amount of reference methyl esters in 10 µl of this solution was determined by liquid scintillation counting as described previously [5,6]. The GLC-analyses were performed as described in [3]. However, the column length and diamter were 4.0 m and 3.18 mm, respectively, in order to improve the resolution of the branched-chain fatty acids. The gas-liquid chromatograph was equipped with a Hewlett-Packard model 3390A electronic integrator. The integrator was programmed with the reference methyl ester values obtained from the liquid scintillation counting, and the amount of each lipid was calculated as one-half of the amount of the remaining methyl esters. However, for glucolipid X the factor one-fourth was used [5]. The reference acyl chains were not degraded or altered in any other way during the TLC and methylating procedures.

When A. laidlawii was grown on the branched-chain fatty acid precursors the membrane lipids were labelled by adding 0.40 mCi/l of [^{3}H] sodium acetate to the medium. The incorporated radioactivity is confined to the acyl chains of the lipid molecules [26]. The lipids were separated by TLC. Each lipid spot was scraped into a Pasteur pipette stoppered with glass wool and the lipids were eluted with methanol-choloroform (9:1, v/v) directly into a scintillation vial. The solvents were evaporated under a stream of warm air. The acyl chain composition was determined in total lipid extracts by evaporating the solvents and treating the residues as described above.

Materials

The straight-chain (purity > 99%) and long branched-chain (purity $\geq$ 98%) fatty acids were obtained from Larodan Fine Chemicals, Malmö, Sweden. Pantetheine (purity 98%) was purchased from Serva Feinbiochemica, Heidelberg, West Germany; 2-methylbutanoic acid (purity 98%) from EGA-Chemie, Steinheim, West Germany; 3-methylbutanoic acid (purity 99%), cholesterol (purity > 99%) and avidin from Sigma Chem. Co., St. Louis, MO., USA. [^{3}H]sodium acetate was from New England Nuclear, Dreieich, West Germany, and [^{3}H]cholesterol and [^{14}C]linoleic acid from Amersham International plc, Amersham, England. The Sephadex® G25 Fine gel was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. n-hexane was of spectroscopic grade (Merck, Darmstadt, West Germany), and the other organic solvents were of analytical grade.

RESULTS

Fatty acid growth requirements of *A. laidlawii* strains A and B

Earlier studies have shown that *A. laidlawii* strain B can grow without unsaturated fatty acids [27,28], while strain A has an absolute requirement for an unsaturated fatty acid with sixteen or eighteen carbon atoms [5,21-23], or a cyclopropane acid with seventeen or nineteen carbon atoms [23,24]. When these strains were grown in the medium used by us, only a minute difference between them could be discerned. Due to a deficiency of pantetheine in this medium, *A. laidlawii* exhibits a very low degree of de novo saturated fatty acid synthesis [8]. None of the saturated fatty acids *n*-C_{12} and *n*-C_{14} to *n*-C_{18} were able to support growth of strains A and B for more than one or two cultivations. Of the long branched-chain fatty acids, anteiso-C_{15}, anteiso-C_{17} and iso-C_{15} supported prolonged growth of both strains, while none of the strains grew with the iso-C_{17} supplement more than two cultivations. All combinations of two of the long branched-chain fatty acids sustained growth of both strains. The only difference observed between the strains was the capacity to grow with pantetheine or the branched-chain fatty acid precursors as the only supplement to the medium; strain B gave a very faint growth while strain A failed to grow. However, both strains grew well when the supplements were added together.

A. laidlawii A cultures reached OD_{540} values of 0.7 to 0.8 when the organism was grown at 37°C on the single long branched-chain fatty acids or on different combinations of these acids. Growth at 27°C on the combinations lacking iso-C_{17} resulted in similar OD_{540} values, but the combinations containing iso-C_{17} were less efficient in supporting growth. 27°C was just above the lower growth temperature limit for

A. laidlawii A when it was grown on iso-C_{15}/iso-C_{17}. When the medium was supplemented with iso-C_5 and/or anteiso-C_5, the OD_{540} value of A. laidlawii A cultures in the early stationary phase of growth was 0.5 to 0.7 at 37°C and 0.4 to 0.5 at 27°C.

Lipid composition with long branched-chain fatty acids

A) Polar head group composition. Five polar lipids always occur in the membranes of A. laidlawii strains A and B [5,29]: $Acyl_2GlcGro$, $Acyl_2$-Glc_2Gro, phosphatidylglycerol (PtdGro), and glycerophosphoryl derivatives of $Acyl_2GlcGro$ and $Acyl_2Glc_2Gro$. A sixth polar lipid, glucolipid X (GLX), is synthesized in increasing amounts by the organisms when the fraction of straight saturated acyl chains in the membrane lipids is increased [5-8,30]. GLX has been suggested to be composed of two diglyceride molecules linked to one glucose molecule [5,31].

Growth on anteiso-C_{15}, anteiso-C_{17} and iso-C_{15} resulted in marked differences in the ratio $Acyl_2GlcGro/Acyl_2Glc_2Gro$ (Table 1); the highest value was obtained with the iso acid supplement. The ionic lipid fraction of the membranes (PtdGro and glycerophosphoryl derivatives of $Acyl_2GlcGro$ and $Acyl_2Glc_2Gro$) varied considerably, and this quantity as well reached the highest level for iso-C_{15}. Trace amount synthesis of GLX was achieved with the two C_{15} acids, while anteiso-C_{17} elicited a higher degree of synthesis (Table 1).

When the long branched-chain fatty acids were supplied in pairs, the changes in the above mentioned quantities were similar to those obtained with the single fatty acids (Table 2). The ratio $Acyl_2GlcGro$/ $Acyl_2Glc_2Gro$ had a higher value in cells grown on the two iso acids as compared to cells grown on the two anteiso acids, both at 27 and 37°C. The amounts of ionic lipids and GLX were also higher for the iso acid

pair. Since iso-C_{15} did not result in any appreciable synthesis of GLX (Table 1), iso-C_{17} must have stimulated its synthesis. The picture is somewhat complicated by the results obtained with the two anteiso/iso fatty acid supplements. The values of the ratio $Acyl_2GlcGro/Acyl_2Glc_2Gro$ did not fall between those produced by the two anteiso and two iso acids. At 37°C anteiso-C_{15}/iso-C_{15} yielded a slightly lower ratio than did anteiso-C_{15}/anteiso-C_{17}, while anteiso-C_{17}/iso-C_{17} yielded a much higher ratio than did iso-C_{15}/iso-C_{17}. At 27°C, however, the ratio was higher with anteiso-C_{15}/iso-C_{15} than with the two anteiso acids. Growth on the two mixtures of anteiso and iso acids resulted in a small reduction of the ionic lipid fraction as compared to growth on the two anteiso acids. GLX synthesis was stimulated by the C_{17} acids but not by the C_{15}acids.

When the growth temperature was lowered from 37 to 27°C there was a marked increase in the $Acyl_2GlcGro/Acyl_2Glc_2Gro$ ratio, but no uniform tendency was noticed in the ionic lipid fraction (Table 2). The effect of incorporation of cholesterol into the membrane was an increase in the $Acyl_2GlcGro/Acyl_2Glc_2Gro$ ratio, except for the iso acid pair supplement where practically no change was obtained. Moreover, the amounts of ionic lipids were raised with 3 to 4 mol%.

B) Acyl chain composition. A very effective incorporation of the fatty acids into the membrane lipids was achieved without using inhibitors of the de novo fatty acid synthesis [18,32], and the branched acyl chains constituted between 95 and 98 mol% of the total amount of acyl chains. The fatty acids were neither elongated nor degraded to shorter chain lengths as judged by the GLC-analyses. When 0.20 mCi/l of [^{3}H]sodium acetate was added to the medium, no radioactivity was incorporated into the acyl chains.

The incorporation of the two fatty acids in a given pair was in some cases affected by the growth temperature and/or by the incorporation of cholesterol (Table 2). Both the anteiso acid and the iso acid supplements resulted in a C_{17}/C_{15} ratio near unity in the membrane lipids at 37°C. The presence of cholesterol in the membrane did not influence this ratio, but it was greatly reduced by a decrease in the growth temperature when the cells were grown on the two iso acids. Anteiso acids were incorporated to a higher degree than the iso acids when supplied in equal concentrations. This tendency was especially marked with the C_{15} acids. Neither the growth temperature nor the incorporation of cholesterol did affect the ratio iso/anteiso acyl chains in the case with the C_{15} acids, while both factors reduced the ratio when the cells were fed with the C_{17} acids. The acyl chain compositions in total membrane lipid extracts from cells grown on all four long branched-chain fatty acids were also determined (Table 2). By lowering the growth temperature the ratio iso-C_{17}/iso-C_{15} was reduced and the ratio anteiso-C_{17}/anteiso-C_{15} was increased, resulting in practically no net change in the acyl chain length. However, the total ratio iso/anteiso acyl chains was reduced, and the pair iso-C_{17}/anteiso-C_{17} contributed to this reduction to a much larger extent than did the pair iso-C_{15}/anteiso-C_{15}.

When *A. laidlawii* was grown on two long branched-chain fatty acids, the ratio of the acyl chains was different in all polar membrane lipids (Table 3). Two patterns of acyl chain ratios could be discerned: one pattern with supplements containing a fatty acid (iso-C_{17}) that alone did not support growth of the organism, and another pattern with supplements containing fatty acids that both supported growth when supplied alone. When iso-C_{17} was present in the medium, GLX made up about 2 to 3 mol% of the polar lipids (Table 2), and incorporation

of iso-C_{17} into this lipid was extremely high as compared to incorporation of anteiso-C_{17} and iso-C_{15} (Table 3). Next after GLX, iso-C_{17} was directed into PtdGro and $Acyl_2GlcGro$. If anteiso-C_{15} was supplied together with anteiso-C_{17} or iso-C_{15}, GLX made up about 0.5 mol% of the polar lipids, and incorporation of the iso acid or C_{17} acid into this lipid was not extreme. Iso-C_{15} and anteiso-C_{17} were primarily directed into glycerophosphoryldiacyldiglucosylglycerol and $Acyl_2Glc_2Gro$. The difference between the highest and lowest acyl chain ratio obtained with each supplement grew bigger when the content of iso acyl chains in the membrane lipids increased (Tables 2 and 3).

Lipid composition with branched-chain fatty acid precursors

A) Acyl chain composition. Pantetheine must be added to the growth medium used by us if *A. laidlawii* A shall be capable of an appreciable de novo synthesis of straight-chain saturated fatty acids [8]. The pantetheine supplement was necessary also for the synthesis of branched-chain saturated fatty acids from short-chain precursors. A pantetheine concentration of 12 µM was used, which results in maximal stimulation of the fatty acid synthesis [8]. [^{3}H]acetate was incorporated into the membrane lipids when added to the medium.

The branched acyl chains constituted 80 to 87 mol% of the total amount of acyl chains when *A. laidlawii* A was grown on the branched-chain fatty acid precursors. Straight saturated acyl chains made up the remaining fraction, with palmitoyl chains being the predominant ones (Table 4). When the anteiso acid precursor was replaced by the iso acid precursor, increasing amounts of C_{13} acyl chains and decreasing amounts of C_{19} acyl chains were incorporated into the membrane lipids, and at 37°C the chain length parameter ($C_{17}+C_{19}/C_{13}+C_{15}$)

decreased from 1.11 to 0.71 (Table 4). A lower value of the chain length parameter was observed at 27°C as compared to 37°C with all supplements. Moreover, the ratio iso/anteiso acyl chains was decreased by decreasing the growth temperature.

B) Polar head group composition. The membrane lipid composition was affected by the type of precursor added to the medium (Table 5). A higher $Acyl_2GlcGro/Acyl_2Glc_2Gro$ ratio was obtained with the iso fatty acid precursor as compared to the anteiso fatty acid precursor. This ratio assumed a value between the two extremes when the cells were grown on an equimolar mixture of the two supplements. Contrary to the results obtained with the long branched-chain fatty acids, the ionic lipid fraction decreased when iso fatty acids were synthesized instead of anteiso fatty acids. GLX synthesis was stimulated when the iso fatty acid precursor was incorporated into the membrane lipids. A higher amount of GLX was achieved with the anteiso fatty acid precursor than with the anteiso-C_{15}/anteiso-C_{17} supplement. This is probably due to the higher amount of straight saturated acyl chains present in the membrane lipids when the cells were grown on the precursor.

DISCUSSION

Long-chain unsaturated or cyclopropane fatty acids have been found to be essential for growth of *A. laidlawii* strain A. It is shown here that these fatty acids can be replaced by certain long saturated branched-chain fatty acids. Exogenously supplied short branched-chain fatty acids, such as 2-methylbutanoic acid and 3-methylbutanoic acid, can serve as primers for de novo synthesis of long saturated branched-chain fatty acids, and are also able to replace the growth requirement for an exogenous long-chain unsaturated or cyclopropane fatty acid. Hence, the configuration of an unsaturated carbon-carbon bond is not essential for growth, since both the branched-chain and the cyclopropane fatty acids are completely saturated. The difference between strains A and B, as regards their fatty acid growth requirements, appears to be minimal. This resemblance between the strains has also been suggested by Rodwell and Mitchell [33].

In the membranes of *A. laidlawii* strains A and B, the molar ratio $Acyl_2GlcGro/Acyl_2Glc_2Gro$ is reduced when the ratio saturated/cis-unsaturated acyl chains in the lipids is decreased [5,7,8,29]. This regulation mechanism has been explained on the basis of lipid molecular geometry [10,11]. Increased amounts of cis-unsaturated acyl chains will increase the hydrophobic bulkiness of the lipid molecules, and mixtures of $Acyl_2GlcGro$ and $Acyl_2Glc_2Gro$ gradually become more prone to form cubic and reversed hexagonal phases [14,15]. Since $Acyl_2GlcGro$ and $Acyl_2Glc_2Gro$ form reversed hexagonal and lamellar phases, respectively [34], the former lipid destabilizes the lamellar phase. The organism responds to increased incorporation of cis-unsaturated fatty acids by reducing the ratio $Acyl_2GlcGro/Acyl_2Glc_2Gro$. This line of reasoning can be applied to the branched-chain fatty acids as well. Anteiso acids

can not be as closely packed as iso acids [2], and a reduced ratio of iso/anteiso acyl chains is thus expected to increase the hydrophobic bulkiness of lipid molecules [4]. This is supported by the fact that PtdEtn containing a low ratio iso/anteiso acyl chains forms a cubic phase, while PtdEtn with a high ratio iso/anteiso acyl chains forms a lamellar phase under the same conditions [4]. The ratio $Acyl_2GlcGro$/ $Acyl_2Glc_2Gro$ can consequently be predicted to decrease when anteiso acyl chains replace iso acyl chains in the membrane lipids. This prediction was born out in three different experimental approaches (Tables 1,2 and 5). The difference in the glucolipid ratio obtained with the anteiso-C_{15} and anteiso-C_{15}/iso-C_{15} supplements as compared to the anteiso-C_{17} and anteiso-C_{17}/iso-C_{17} supplements, respectively (Table 1 and 2), may also be explained by the packing properties of the acyl chains. When the length of the hydrocarbon chain is increased, the area per branched-chain fatty acid in a monolayer at the water-air interface is reduced [2]. The hydrophobic bulkiness of lipid molecules containing branched acyl chains should thus be reduced by increasing the chain length, and the ratio $Acyl_2GlcGro/Acyl_2Glc_2Gro$ is expected to rise in order to maintain optimal packing of the membrane lipids. Since elongation of branched acyl chains probably supresses the tendency of $Acyl_2GlcGro/Acyl_2Glc_2Gro$ mixtures to form non-lamellar phases, it would be favourable for an organism to synthesize longer branched acyl chains at higher temperatures in order to counteract the bilayer destabilizing effect of the temperature change [15]. Elongation of branched acyl chains at higher growth temperatures has been noticed in *B. megaterium* [3], *B. caldolyticus* [35], *Flavobacterium thermophilum* [36] and *A. laidlawii* (Table 4).

A $10^{o}C$ decrease in the growth temperature resulted in an elevation of the ratio $Acyl_2GlcGro/Acyl_2Glc_2Gro$ (Table 2). This response has also been obtained with A. laidlawii A growing on straight-chain saturated and cis-unsaturated fatty acids [6], and has been interpreted as a response to changes in the lipid molecular shape [10,11]. When cholesterol was incorporated into the membranes, the ratio $Acyl_2GlcGro/Acyl_2Glc_2Gro$ was raised with most branched-chain fatty acid supplements (Table 2), as opposed to the reduction of this ratio when the organism is grown on oleic acid only [7]. These results may be explained by the different steric configurations of oleic acid and branched-chain fatty acids. The double bond introduces a packing disturbance at carbon atoms 9 and 10 in oleoyl chains, which is at the same level as the lower part of the rigid steroid nucleus [37]. The branched C_{15} and C_{17} acyl chains have their packing disturbance at the same level as the hydrocarbon side chain of cholesterol, which exhibits a high degree of mobility in phospholipid bilayers [38]. Cholesterol promotes the formation of a reversed hexagonal phase in dioleoylmonoglucosylglycerol/dioleoyl-diglucosylglycerol mixtures [15], and the glucolipid ratio is consequently reduced when cholesterol is incorporated into A. laidlawii A mebranes enriched in oleoyl chains [10]. It can be anticipated that cholesterol is better accomodated to membrane lipids containing branched acyl chains, and that the sterol molecule exerts a slightly stabilizing effect on a lamellar phase composed of these lipids. Hence, cholesterol has not a general effect on the packing properties of glycerolipids. This is supported by the finding that cholesterol lowers the temperature for the transition from a lamellar to a reversed hexagonal phase in mixtures with dioleoylphosphatidylethanolamine, while this transition temperature is increased when cholesterol is mixed with PtdEtn containing large amounts of polyunsaturated acyl chains [39].

A. laidlawii strains A and B reduce the amount of ionic lipids in their membranes when the ratio saturated/cis-unsaturated straight acyl chains present in the lipids is increased [5,7,8,29]. Hence, when the lateral packing area of the lipid molecules decreases, the ionic lipid fraction is reduced. This regulation may be governed by an aim to conserve the surface charge density of the membrane [10]. Since anteiso fatty acids are more loosely packed than iso fatty acids [2], it can be expected that the ionic lipids are synthesized in smaller amounts when A. laidlawii is grown on the latter supplement. This was actually the case when the branched-chain fatty acid precursors were used (Table 5). Partly diverging results were obtained when the organism was fed with the long branched-chain fatty acids. However, changes in the same direction of the values of the ratio $Acyl_2GlcGro$/ $Acyl_2Glc_2Gro$ and the amount of ionic lipids have been observed under other growth conditions as well. Replacement of sodium ions by lithium ions in the growth medium, leads to an increase in both of the above mentioned quantities when A. laidlawii A is grown on oleic acid (A. Christiansson and Å. Wieslander, unpublished results). The ionic lipid fraction was increased upon cholesterol incorporation into the membrane (Table 2). The same response is achieved when the cells are grown on oleic acid [7], and regulation of the membrane surface charge density has been suggested to be the cause [10].

It is evident from this investigation that the iso-C_{17} fatty acid is unfavourable as a membrane lipid constituent at temperatures within the mesophilic growth range. Iso-C_{17} alone does not support growth of A. laidlawii, and when the organism is grown on different combinations of long-chain branched fatty acids, the ratios iso-C_{17}/iso-C_{15} and iso-C_{17}/anteiso-C_{17} are greatly reduced by lowering the growth temperature,

while the ratios anteiso-C_{17}/anteiso-C_{15} and iso-C_{15}/anteiso-C_{15} are either slightly affected or not affected at all. Iso-C_{17} is however useful in the thermophilic growth range [3], and the fraction of this acyl chain can rise to between 50 and 65 mol% in membranes of extreme thermophiles [35,36]. The temperature-induced regulation of the ratio iso/anteiso acyl chains can be parallelled with the regulation of the ratio saturated/cis-unsaturated straight acyl chains. A temperature change alters the molecular geometry of lipids containing either kind of acyl chains [4,15], and a decrease in the above mentioned ratios upon a reduction of the growth temperature probably restores the shape of the lipid molecules, and hence the bilayer stability [10].

The genus Bacillus synthesizes the branched-chain fatty acid precursors from valine, leucine and isoleucine, and the precursors are elongated via the malonyl-coenzyme A pathway [1]. A. laidlawii utilizes the same pathway for chain elongation [40], and growth of this organism on the branched-chain fatty acid precursors is consequently a good imitation of the circumstances prevailing in the Bacillus membranes. Both organisms reduce the average acyl chain length and the ratio iso/anteiso acyl chains when the growth temperature is lowered (Table 4) [3]. These regulation mechanisms seem to be general, and they thus reflect the physico-chemical properties of the branched acyl chains.

When A. laidlawii strains A and B are grown on mixtures of palmitic and oleic acid, the acyl chain ratio in the different polar membrane lipids exhibits substantial variations [5,30]. The ratio palmitoyl/oleoyl chains is higher than the average in GLX and $Acyl_2GlcGro$. If iso-C_{17} is present together with iso-C_{15} or anteiso-C_{17} in the growth medium, iso-C_{17} is preferentially incorporated into GLX, $Acyl_2GlcGro$ and PtdGro. Iso-C_{17} and n-C_{16} have two common properties in this context: (1) they are the highest-melting fatty acids in the three

supplements discussed; and (2) they fail to support growth of *A. laidlawii*. The difference between the highest and lowest acyl chain ratio obtained with each supplement, increases when the average ratio palmitoyl/oleoyl chains as well as the average ratio iso/anteiso acyl chains in the lipids increases (Table 3) [30]. GLX incorporates extreme amounts of iso-C_{17}, a fact supporting the suggestion put forward earlier [8], that GLX acts as a scavenger lipid withdrawing diglycerides with extreme acyl chain compositions. Thus, each one of the lipid synthesizing enzymes selects a proper substrat, which results in the maintenance of a carefully controlled acyl chain composition of the different lipid species.

CONCLUSION

It is shown here that the polar head group composition of the *A. laidlawii* A membrane lipids is influenced by the type of branched-chain fatty acid that is incorporated into the membrane. This connection between polar head group composition and acyl chain composition also exists when the organism is grown on straight-chain saturated and cis-unsaturated fatty acids [10]. Moreover, alterations in the incorporated ratios of iso/anteiso acyl chains and palmitoyl/oleoyl chains yield the same qualitative response in polar head group composition. The relative amounts of polar head groups of different sizes and charges are thus similarly regulated by two kinds of changes in the steric configuration of the acyl chains. The ratios iso/anteiso acyl chains and palmitoyl/oleoyl chains of the membrane lipids, are altered in the same direction upon changes in the growth temperature. These findings support our earlier statement that the *effective* shape of the lipid molecules governs the regulation of the lipid composition in *A. laidlawii* A membranes [10,11].

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Table 1. Polar head group composition of membrane lipids from *Acholeplasma laidlawii* A grown on long-chain branched fatty acids. The growth medium was supplemented with 120 μM of the fatty acid, and cells were grown at 37°C.

Fatty acid supplement[a]	$Acyl_2GlcGro/Acyl_2Glc_2Gro$[b]	Mol% ionic lipids[c]	Mol% GLX[d]
a-C_{15}	0.86	31.1	0.5
a-C_{17}	1.09	34.0	1.6
i-C_{15}	1.55	38.9	0.5

[a] *a*: anteiso, *i*: iso, C_n: number of carbon atoms

[b] Molar ratio diacylmonoglucosylglycerol/diacyldiglucosylglycerol

[c] Ionic lipids: phosphatidylglycerol, glycerophosphoryldiacylmonoglucosylglycerol and glycerophosphoryldiacyldiglucosylglycerol

[d] GLX: glucolipid X

Table 2. Polar head group and acyl chain composition of membrane lipids from *Acholeplasma laidlawii* A grown on different combinations of long-chain branched fatty acids. The total concentration of fatty acids in the growth medium was 120 μM, and the fatty acids were supplied in equimolar concentrations. Cholesterol was added to a final concentration of 20 μM. Cells were grown at 27°C and 37°C.

Fatty acid supplement[a]	Growth temperature (°C)	$Acyl_2GlcGro/Acyl_2Glc_2Gro$[b]	Mol% ionic lipids[b]	Mol% GLX[b]	C_{17}/C_{15}[c]	Iso/Anteiso[d]
a-C_{15}/a-C_{17}	37	0.91	35.2	0.4	1.01	-
a-C_{15}/a-C_{17}/chol	37	1.00	38.9	0.6	1.00	-
a-C_{15}/a-C_{17}	27	2.34	35.1	0.5	1.01	-
a-C_{15}/i-C_{15}	37	0.80	33.6	0.6	-	0.59
a-C_{15}/i-C_{15}/chol	37	0.96	36.4	0.4	-	0.57
a-C_{15}/i-C_{15}	27	3.17	34.9	0.4	-	0.58
a-C_{17}/i-C_{17}	37	2.39	34.0	1.7	-	0.93
a-C_{17}/i-C_{17}/chol	37	3.38	33.8	1.9	-	0.55
a-C_{17}/i-C_{17}	27	3.46	31.2	3.3	-	0.40
i-C_{15}/i-C_{17}	37	1.45	39.2	2.6	1.11	-
i-C_{15}/i-C_{17}/chol	37	1.40	42.0	3.2	1.11	-
i-C_{15}/i-C_{17}	27	3.05	41.7	2.5	0.51	-
a-C_{15}/a-C_{17}/i-C_{15}/i-C_{17}	37	-	-	-	1.13	1.07
a-C_{15}/a-C_{17}/i-C_{15}/i-C_{17}	27	-	-	-	1.11	0.68

[a] a: anteiso, i: iso, C_n: number of carbon atoms, chol: cholesterol [b] See footnote to Table 1;

[c] Molar ratio C_{17}/C_{15} acyl chains; [d] Molar ratio iso/anteiso acyl chains

Table 3. Acyl chain ratios in the polar membrane lipids from Acholeplasma laidlawii A grown on long-chain branched fatty acids supplied in pairs. The total concentration of fatty acids in the growth medium was 120 μM, and the fatty acids were supplied in equimolar concentrations. Cells were grown at 37°C. The acyl chain ratios are normalized, i.e. the value obtained for each lipid from the GLC-analysis has been divided by the average value obtained for all polar membrane lipids (Table 2).

Lipid	Molar acyl chain ratios[a]			
	a-C_{17}/a-C_{15}	i-C_{15}/a-C_{15}	i-C_{17}/a-C_{17}	i-C_{17}/i-C_{15}
Glycerophosphoryl-diacyldiglucosylglycerol	1.40	1.66	0.40	0.57
Glycerophosphoryl-diacylmonoglucosylglycerol	0.76	0.93	0.58	0.37
Phosphatidylglycerol	0.90	0.50	1.27	2.12
Diacydiglucosylglycerol	1.17	1.28	0.60	0.74
Diacylmonoglucosylglycerol	0.83	0.96	1.23	0.93
Glucolipid X	1.37	0.67	6.1	21.4

[a] See footnote to Table 1.

Table 4. Acyl chain composition of total membrane lipid extracts from Acholeplasma laidlawii A grown on branched-chain fatty acid precursors. Pantetheine was added at a concentration of 12 μM, the fatty acid precursors at 1.0 mM when supplied alone, and at 0.5 mM each when supplied together. Cells were grown at 27°C and 37°C.

Acyl chain[a]	Fatty acid precursor[a], growth temperature					
	a-C_5 37°C	a-C_5 27°C	a-C_5/i-C_5 37°C	a-C_5/i-C_5 27°C	i-C_5 37°C	i-C_5 27°C
n-C_{12}	0.2	0.1	0.2	0.1	0.3	0.2
i-C_{13}	-	-	1.9	1.5	7.2	6.8
a-C_{13}	0.8	0.9	0.6	0.8	-	-
n-C_{14}	1.0	1.2	1.1	1.3	1.6	2.3
i-C_{15}	-	-	17.9	18.3	42.8	44.4
a-C_{15}	39.5	42.0	24.3	27.3	-	-
n-C_{15}	0.6	0.7	0.5	0.5	0.5	0.6
n-C_{16}	9.7	12.0	9.3	10.6	9.4	12.6
i-C_{17}	-	-	24.6	20.9	35.5	29.2
a-C_{17}	42.1	37.9	15.7	15.6	-	-
n-C_{17}	0.8	0.5	0.4	0.3	0.4	0.4
n-C_{18}	2.7	2.9	3.0	2.4	2.3	2.4
a-C_{19}	2.6	1.8	0.5	0.4	-	-
Acyl chain ratios						
$C_{17}+C_{19}/C_{13}+C_{15}$[b]	1.11	0.93	0.91	0.77	0.71	0.57
Iso/Anteiso[c]	-	-	1.08	0.92	-	-

[a] n: normal, i: iso, a: anteiso, C_n: number of carbon atoms

[b] Molar ratio of $(C_{17}+C_{19})/(C_{13}+C_{15})$ branched acyl chains

[c] Molar ratio iso/anteiso acyl chains

Table 5. Polar head group composition of membrane lipids from *Acholeplasma laidlawii* A grown on branched-chain fatty acid precursors. Pantetheine was added at a concentration of 12 μM, fatty acid precursors at 1.0 mM when supplied above, and at 0.5 mM each when supplied together. Cells were grown at 37°C.

Fatty acid precursor[a]	$Acyl_2GlcGro/Acyl_2Glc_2Gro$[a]	Mol% ionic lipids[a]	Mol% GLX[a]
a-C_5	0.99	33.8	1.3
a-C_5/i-C_5	1.16	30.5	3.5
i-C_5	1.35	23.4	3.0

[a] See footnotes to Table 1.

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